Disrupting insect-mediated transmission of plant viruses

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This dissertation is submitted for the degree of
Doctor of Philosophy

Corpus Christi College May 2019

Dedicated to my family for being the support in all my adventures.
¡Gracias a ustedes cada día quiero ser mejor!

A Tomas y Carla por ser la fuente de sonrisas

Declaration

I hereby declare that the contents of this dissertation is the result of my own work except where specific reference is made to the work of others or work done in collaboration with others. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared or specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University of similar institution except as specified in the text. The dissertation does not exceed the maximum length permitted by the Board of Graduate Studies

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May 2019

Summary

Many plant viruses are vectored by aphids in the non-persistent mode, in which virus particles are transported on these insects' specialised piercing mouthparts (stylets). Virus infection can change plant-vector interactions and it is thought that this may accelerate virus transmission. To better understand how to inhibit virus transmission, I studied vectoring of cucumber mosaic virus (CMV) and turnip mosaic virus (TuMV) between *Arabidopsis thaliana* plants by the aphid *Myzus persicae*. Among other things, I investigated if there were differences in the extent to which the viruses modified aphid-plant interactions in different Arabidopsis accessions, as well as the intrinsic susceptibility of these accessions to aphid infestation.

A range of Arabidopsis accessions showed differences in attractiveness and susceptibility to aphids and variations in virus-induced changes in plant-aphid interactions. I found that aphids were attracted to plant volatiles emitted by CMV-infected Col-0 and CMV-infected Ei-2 plants. Interestingly, aphids settle more readily on CMV-infected Ei-2 plants than CMV-infected Col-0 plants. However, aphids did not prefer to settle on CMV-infected plants of either accession. Thus, I used these accessions to determine how to manipulate aphid behaviour to inhibit virus transmission using two types of microcosm: simple lines of plants (with various mixtures of the two accessions) and two-dimensional arrays ('fields') of plants. The simple line experiments showed that aphid-mediated transmission could be disrupted using mixtures of accessions with differences in intrinsic aphid attractiveness and susceptibility to aphid infestation. In the two-dimensional 'field' experiments, two approaches, which included CMV- and TuMV-resistant plants were most effective in inhibiting virus transmission. Resistant plants in both Arabidopsis backgrounds were either mixed in various proportions (resistant v. susceptible and Col-0 v. Ei-2) in randomised planting layouts or with plants arranged as barriers. A 33% proportion of virus-resistant plants randomly distributed was sufficient to inhibit virus transmission. A barrier of Ei-2 CMV-resistant plants or TuMV-resistant plants in a population of Col-0 plants reduced virus transmission of CMV and TuMV. The latter approach retained more aphids than populations of solely Col-0 plants. Thus, Ei-2 plants can be used as trap plants to attract and 'sanitise' viruliferous aphids by inducing them to deposit virus particles in resistant plants, thus inhibiting onward transmission. The results show that there is potential for using intraspecific variation in host plants to inhibit aphid-mediated transmission.

ACKNOWLEDGEMENTS

I am extremely thankful to my supervisor, Prof. John Carr, for his support and guidance not only on my PhD studies but also in my professional development. I would also like to thank Dr. Alex Murphy for her endless technical support and advice in the execution of my experiments. I am extremely grateful to Adrienne Pate for her enthusiastic support and technical capability. Thanks to Dr. Trisna Tungadi and Dr. Ruairi Donnelly for the scientific support and helpful discussions. I am very grateful to Dr. Matt Davey for his help with the GC-MS experiments. Thank you also to Dr. Nik Cunniffe for his help with statistical analysis.

My time in the lab would not have been fun without all the people in the Carr Lab. Thanks for the ones that left Dr. Jacqui Poon (for your friendship), Dr. Francis Wamonje (for being my uncle at College and aphid expert in the lab), Dr. Sanjie Jiang (for your friendship and technical support in my VOC experiments). The ones still in the lab with whom I have shared invaluable experiences, Dr. Sun-Ju Rhee, Dr. Nina Lukhovitskaya, Deusa Abreu, Lewis Watt, Netsai Mhlanga, Ebenezer Gyamera, Anna Platoni and Warren Arinaitwe. Thank you to my friends in the Department of Plant Sciences, especially, Gabriela Doria and Alfonso Timoneda.

I could have not gotten here without the support of my beloved family and friends. Thank you to my parents Nohemi and Livio for always cheering me to pursuit my dreams. Thank you to my siblings Paulina and Daniel who knew how to lift me up when I needed the most. ¡Gracias por estar en las buenas y en las peores! A big “Thank you” to my friends back in Ecuador and all over the world. Our friendship has no borders.

I would like to acknowledge SENESCYT for providing me with the financial support for my PhD.

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List of abbreviations

4-methoxy-indol3yl-methylglucosinolate (**4MI3M**)
6-benzyl amino purine (**BAP**)
artificial microRNA (**amiRNA**)
avirulence (**Avr**)
barley yellow dwarf virus (**BYDV**)
cauliflower mosaic virus (**CaMV**)
coat protein (**CP**)
clustered regulatory interspaced short palindromic repeats (**CRISPR**)
cucumber mosaic virus (**CMV**)
cylindrical inclusion protein (**CI**)
days post inoculation (**dpi**)
dicer-like proteins (**DCL**)
double stranded RNA (**dsRNA**)
ethylenediaminetetraacetic acid (**EDTA**)
electrical penetration graph (**EPG**)
enzyme-linked immunosorbent assay (**ELISA**)
ephrin receptor (**EpH**)
eukaryotic translation initiation factor (**eIF**)
gas chromatography (**GC**)
general linear model (**GLM**)
green fluorescent protein (**GFP**)
hairpin (**hp**)
helper component proteinase (**HC-Pro**)
hypersensitive response (**HR**)
left border (**LB**)
least significant difference (**LSD**)
mass spectrometry (**MS**)
mean relative growth rate (**MRGR**)
microRNAs (**miRNAs**)
Murashige and Skoog agar (**MSA**)
naphtalene acetic acid (**NAA**)
nuclear Inclusion a-Protease (**NIaPro**)
nuclear Inclusion b-Protease (**NIbPro**)
open reading frame (**ORF**)
optical density (**OD**)
PAMP-triggered immunity (**PTI**)
pathogen associated molecular patterns (**PAMPS**)
pattern recognition receptors (**PRRs**)
potato leafroll virus (**PLRV**)
potato virus Y (**PVY**)
pretty interesting *Potyviridae* ORF(**PIPO**)
principal component analysis (**PCA**)
polytetrafluoroethylene (**PTFE**)
right border (**RB**)
RNA interference (**RNAi**)
RNA-induced silencing complex (**RISC**)
short interference RNA (**siRNA**)
single stranded RNA (**ssRNA**)
Tris-acetate EDTA (**TAE**)
tobacco mosaic virus (**TMV**)
turnip mosaic virus (**TuMV**)
turnip yellows mosaic virus (**TYMV**)
untranslated region (**UTR**)
volume/volume (**v/v**)
volatile organic compounds (**VOCs**)
viral suppressor of RNA silencing (**VSR**)
virus protein genome-linked (**VPgs**)
volatile organic compound (**VOC**)
viral protein-genome linked (**VPg**)
weight/volume (**w/v**)
wild-type (**WT**)

Chapter 1 Introduction

1.1 Background

An important challenge for ensuring increased crop productivity is control of diseases and pests. It is estimated that demand for food will double over the period from 2005 to 2050 (Tilman et al., 2011). Losses in crop yield and quality are caused by a number of pathogens and pests including bacteria, fungi, oomycetes, nematodes, insects and viruses. Plant virus diseases represent an increasing threat to food security due to climate change (Jones, 2014b). Most plant viruses use insect vectors for transmission. Climate change is affecting the ranges of these insects as higher temperatures allow higher rates of growth and reproduction (Anderson et al., 2004). Expanding human population is increasing the acreage of farmland and disturbing natural plant communities; thus, new plant diseases may become capable of invading formerly new areas (Jones, 2014b). It is estimated that viruses cause half of the emerging plant diseases (Anderson et al., 2004). Potential methods to mitigate losses caused by plant viruses include the breeding of resistant plants, attenuation of pathogen transmission, and promotion of genetic diversity in the crop (Strange and Scott, 2005; Worrall et al., 2015; Groen et al., 2017).

The knowledge gained from studying virus-host-vector interactions in model plants such as *Arabidopsis thaliana* and *Nicotiana tabacum* (Ziebell et al., 2011; Westwood et al., 2013a; Tungadi et al., 2017) might contribute to novel methods for mitigating viral diseases of crops. This may be especially useful for crops in sub-tropical or tropical countries where vector densities are often higher and in developing countries, where inputs such as pesticides are in short supply or expensive. For this reason the work I have carried out, and which is described in this thesis, is part of a

wider research programme of studies to protect common bean (*Phaseolus vulgaris*) in East and Central Africa. In this region the crop is under threat due to aphid-transmitted viral diseases (Worrall et al., 2015).

1.2 Vector-mediated transmission of plant viruses

Most vectored plant viruses are transmitted by fungi, nematodes, mites and insects (Ng and Falk, 2006). Insect vectors include aphids, leafhoppers, whiteflies and thrips, but aphids are the most frequently encountered vectors of plant viruses. Virus transmission comprises several phases including host-location, acquisition of virus, transport and inoculation of virus to new hosts (Ng and Falk, 2006; Brault et al., 2010). Insect-mediated transmission of plant viruses is classified into three modes of transmission: persistent, semi-persistent, and non-persistent. Persistently transmitted viruses are retained for the vector's entire life span. Virus particles must pass through the gut, circulate within the hemocoel and reach the salivary glands for successful transmission. Viruses that replicate within the vector cells are described as propagative and those that do not replicate in the vector are termed non-propagative. For both non-persistent and semi-persistent transmission insects remain viruliferous (i.e virus-bearing and infectious to the plant host) for short periods of time that last for minutes or hours (Ng and Falk, 2006). Semi-persistently transmitted viruses are retained for hours or days (Ng and Falk, 2006) but lost after vector moulting, whereas non-persistently transmitted viruses are loosely attached to the tip of piercing mouthparts (stylets) of the insect, and are not internalized by the insect (Pirone & Perry, 2002). Vectors land on the plant and probe the epidermal cells to assess host suitability (Section 1.5.2). The capability to spread viruses efficiently is linked to aphids' capacity to sample cell contents by making intracellular probes without killing the cell. The mandibular and maxillary stylets form a fine needle-like structure that penetrates plant cell walls without causing extensive damage to plant tissues. Aphid stylets perform the sampling of host cells to assess

host suitability (Powell et al., 2006) (see Section 1.5.2). Probing usually takes less than 30 seconds and is the optimal process for acquisition or inoculation of non-persistently transmitted viruses (Powell, 2005). Particles of non-persistently transmitted viruses remain bound to the stylets for 2-4 hours and are acquired from and inoculated into epidermal host cells (Krenz et al., 2015; Powell, 2005). Inoculation of non-persistently transmitted viruses occurs during the first phase of the feeding process, when watery saliva is injected into the cytoplasm of the plant cell (Martín et al., 1997). Non-persistently transmitted viruses are transmitted more efficiently by aphids that are starved prior to virus acquisition (Powell, 1993; Ng and Perry, 1999).

1.3 Cucumber mosaic virus

Cucumber mosaic virus (CMV) is a positive-sense single-stranded RNA virus that is the type species of the *Cucumovirus* genus in the family Bromoviridae (Palukaitis & García-Arenal, 2003). CMV-induced diseases have been reported in more than 1000 species in over a hundred families of monocots and dicots (Roossinck, 2002; Mochizuki & Ohki, 2012). CMV is transmitted in a non-persistent manner by at least 75 aphid species, including *Myzus persicae* (Nouri et al., 2014). CMV can also be transmitted mechanically and via seed in a few plants such as common bean (Jacquemond, 2012). Recently, it was confirmed for CMV that the initial host cells inoculated by the insect vector are the epidermal cells during the initial stages of probing (Krenz et al., 2015).

1.3.1 CMV strains

Most CMV strains can be categorised into Subgroups IA, IB or II based on RNA sequence similarity (Roossinck, 2002). Subgroup IA and IB strains often exhibit more severe symptoms than Subgroup II CMV strains but this is host-specific (Wahyuni et al., 1992). For example in tobacco, *Nicotiana benthamiana,* and *Arabidopsis* accession Col-0, CMV strain Fast New York (Fny-CMV, subgroup IA) generally

causes severe symptoms, whereas CMV strain *Lactuca sativa* (LS-CMV, subgroup II) is milder (Lewsey et al., 2007, 2009).

1.3.2 The CMV genome

CMV has three genomic RNA segments packaged in separate particles (virions), with capsid shells approximating T=3 icosahedra formed from 60 coat protein (CP) molecules that form virions with a size of 28 nm (Palukaitis and García-Arenal, 2003). CMV encodes five known proteins (Figure 1.1) (Palukaitis and García-Arenal, 2003). The 1a and 2a proteins are components of the viral replicase complex and can also influence viral movement. The 1a protein is translated directly from RNA 1 and contains helicase and methyltransferase domains. The 2a protein is translated directly from the 5' proximal open reading frame of RNA 2. The 2a protein is an RNA-dependent RNA polymerase that is required for the replication of genomic RNAs and transcription of sub-genomic RNAs (Cillo et al., 2002; Palukaitis and García-Arenal, 2003; Seo et al., 2009). The 1a and 2a proteins form a replicase complex that is located in the tonoplast of infected cells, where virus replication is suggested to take place (Cillo et al., 2002).

The 3a movement protein is required for viral movement between cells through plasmodesmata and longer distance movement through the phloem (Ding et al., 1995; Hwang et al., 2007). The movement protein is directly translated from RNA 3 (Palukaitis and García-Arenal, 2003; Jacquemond, 2012). The CP is encoded by RNA 3 but expressed from sub-genomic RNA 4 (Figure 1.1) (Palukaitis and García-Arenal, 2003; Jacquemond, 2012). The CP, together with the movement protein, is required for cell-to-cell movement (Canto et al., 1997). The CP is not only crucial for encapsidation but also determines the binding of virus particles to the aphid vector mouthparts and is therefore essential for transmission by aphids (Chen and Francki, 1990). Specific amino acid residues within the CP determine virion binding to

unknown receptors in the aphid stylet during feeding (Liu et al., 2002). The tip of aphid maxillary stylets has an organ called acrostyle, which harbours receptors of noncirculative viruses such as CMV (Webster et al., 2018).

The 2b protein is a small (12 kDa) polypeptide translated from sub-genomic RNA 4A (Ding et al., 1994) (Figure 1.1). The 2b protein has many functions including suppression of the potent antiviral mechanism, RNA silencing (Section 1.6.1). It was one of the first identified viral suppressors of RNA silencing (VSR) (Béclin et al., 1998). Its ability to inhibit RNA silencing is due to its ability to bind small RNAs and not to its ability to localise to the nucleus or interact with Argonaute (AGO) proteins (González et al., 2010), as thought previously (Zhang et al., 2006). In the host cell, the 2b protein localises predominantly to the nucleus, but occurs also in the cytoplasm although the exact distribution differs considerably for 2b proteins encoded by strains of different subgroups (Lucy et al., 2000; Du et al., 2014). The 2b protein is also important for virus systemic movement and symptom induction (Ding et al., 1994; Lewsey et al., 2009). The 2b protein also influences cell-to-cell movement and the distribution of CMV between different cell types (Soards et al., 2002). The 2b protein molecules present in the cytoplasm are required for antiviral silencing activity. However, 2b protein localised in the nucleus/nucleolus enhances CMV virulence (Du et al., 2014). The 2b protein is also involved in host-vector interactions (see Section 1.9.2).

1.4 The Potyvirus genome

The members of the genus *Potyvirus* have similar genome organisation and gene expression mechanisms (Revers and García, 2015) (Figure 1.2). The virions of potyviruses are flexuous rods encapsidating a positive sense single stranded RNA genome (Ivanov et al., 2014). The virions are 750 nm in length and constructed from

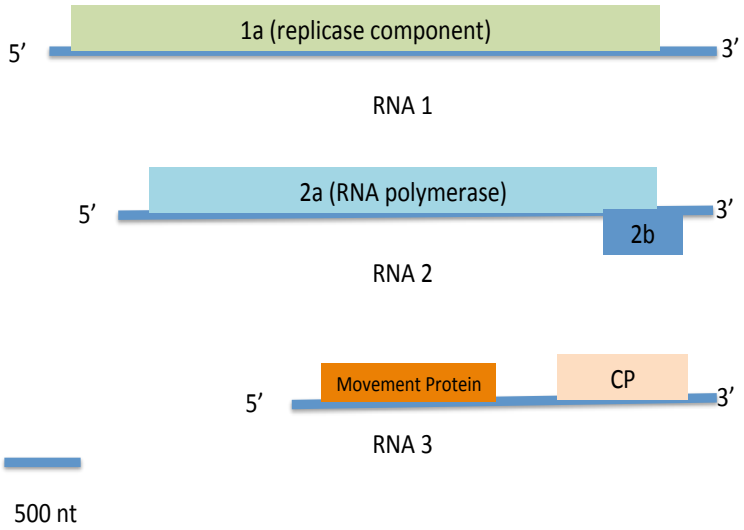


Figure 1.1 Diagram of CMV genome structure

The open reading frames (ORF) are represented as boxes. RNA 1 encodes the protein 1a, which is a replicase component that has a methyltransferase domain within its N-proximal and a helicase motif in the C-proximal region. RNA 2 encodes the 2a protein and the 2b protein from an ORF overlapping the 3'-terminal part of the 2a ORF. The 2b protein is translated from sub-genomic RNA 4A. RNA 3 is also bicistronic and encodes the 3a, movement protein, and coat protein (CP). The coat protein is translated from sub-genomic RNA 4.

~ 2,000 CP molecules that are arranged helically around the genomic RNA. Several potyviral proteins are known to be multifunctional.

The 5'-terminus of the viral RNA has a covalently attached virus-encoded VPg protein (viral protein-genome-linked) and the 3'-terminus has a poly(A) tail. Potyviral VPgs are involved in virus replication and viral RNA translation (Ivanov et al., 2014). VPgs interact with the eukaryotic translation initiation factors, eIF4E, eIF(iso)E, or eIF4G to allow translation of the genomic RNA by host ribosomes (Eskelin et al., 2011).

The potyviral genomic RNA contains an open reading frame (ORF) coding for a polyprotein that is processed by virus-coded proteinases into 11 mature proteins (Walsh and Jenner, 2002; Revers and García, 2015). The 11 mature proteins include P1, helper-component proteinase (HC-Pro), P3, 6K1, cylindrical inclusion, 6K2, nuclear inclusion a (NIa), which is further processed to produce VPg and NIaPro, nuclear inclusion b (NIb), CP and P3N-PIPO. The recently described P3N-PIPO movement protein is a fusion of a short ORF, pretty interesting *Potyviridae* ORF (PIPO), with the N terminal region of P3 protein (Chung et al., 2008). It is translated from variants of viral RNA that are produced at a low rate (<2%) during replication by insertion of an additional "A" in a conserved GAAA sequence. The insertion changes the translation reading frame and allows P3N-PIPO to be expressed (Wei et al., 2010; Olspert et al. 2015 and 2016;)

The potyviral P1 protein is a serine protease that cleaves at its own C-terminus (Verchot et al., 1991). It stimulates genome amplification and enhances virus infection (Verchot and Carrington, 1995). In some potyviruses, the P1 protein function may enhance or act as VSR. For example, cassava brown streak virus lacks HC-Pro and P1 acts as a silencing suppressor, whereas in potato virus Y

(PVY), P1 may enhance the RNA silencing function of HC-Pro (Mbanzibwa et al., 2009; Tena Fernández et al., 2013).

HC-Pro is a cysteine protease that self-cleaves at its C-terminus. In some potyviruses it has VSR activity although in others the VSR may be the P1-HC-Pro fusion, P1, VPg, or P1N-PISPO (Valli et al., 2006; Rajamäki and Valkonen, 2009; Rajamäki et al., 2014; Untiveros et al., 2016). HC-Pro VSR activity appears to work by sequestration of siRNAs (Lakatos et al., 2006) (RNA silencing is discussed in Section 1.6.1). HC-Pro is required to stabilize CP and facilitates long distance movement and maintenance of genome replication (reviewed by Revers and Garcia, 2015). The N-terminal domain is required for aphid transmission (Govier et al., 1977).

The P3 protein is important for viral replication and viral pathogenicity (reviewed by Revers and Garcia, 2015). Some potyviruses also produce a VSR called P1N-PISPO ("Pretty interesting sweet potato potyvirus ORF") by the same mechanism as P3N-PIPO (Olspert et al., 2015; Untiveros et al., 2016).

The cylindrical inclusion protein (CI) has ATPase and RNA helicase activities, which are essential for virus RNA replication (Fernández et al., 1997). CI protein together with P3N-PIPO aid virus movement (Wei et al., 2010). NIaPro is the protease responsible for the proteolytic processing of the central and C-terminal regions of the polyprotein (Adams et al., 2005). NIaPro is important for vector-host interactions as this protein may alter ethylene signalling to enhance aphid performance on TuMV-infected plants (Casteel et al., 2014, 2015; Bak et al., 2017) (see Section 1.9.2).

NIbPro is the potyviral RNA-dependent RNA polymerase. It contains the conserved GDD motif characteristic of RNA-dependent RNA polymerases found in diverse plant and animal viruses (Kamer and Argos, 1984). The 6K2 protein has a central

hydrophobic domain that is associated with VPg-NIaPro and is localised in ER-derived membranes that form cytoplasmic vesicles, which are viral replication sites (Revers and Garcia, 2015).

The CP encapsidates the viral genome and is essential for cell-to-cell virus movement as well as systemic movement (Revers and García, 2015). The CP N-terminal contains a conserved amino acid motif (DAG) which is involved in the binding of the coat protein to HC-Pro which binds to putative receptors in aphid stylets (Pirone and Blanc, 1996). The CP:HC-Pro interaction is flexible as HC-Pro from one potyvirus can interact with the CP of another potyvirus. For example, Bean yellow mosaic HC-Pro facilitated aphid transmission of Potato yellow virus (PYV) (Pirone and Blanc, 1996). Thus, HC-Pro can aid the aphid transmission of another potyvirus. HC-Pro can also determine specificity of transmission by different aphid species (Wang et al., 1998).

1.4.1 Turnip mosaic virus

Turnip mosaic virus (TuMV) is a species in the genus *Potyvirus* in the family *Potyviridae*. TuMV has a positive single stranded RNA of 9830 nucleotides. TuMV is also a major virus infecting Brassicaceae (Walsh and Jenner, 2002). Aphids are considered the main vector of TuMV whose transmission is in a non-persistent manner (Section 1.2). The ability to infect Arabidopsis makes TuMV an excellent tool to identify resistance genes and to study plant-virus interactions (Walsh and Jenner, 2002). TuMV strain UK-1 infects a broad range of Arabidopsis accessions (Martín et al., 1999). Although resistance genes to bacteria, fungi and other viruses have been described in Arabidopsis, there is a lack of resistance genes to TuMV (Walsh and Jenner, 2002).

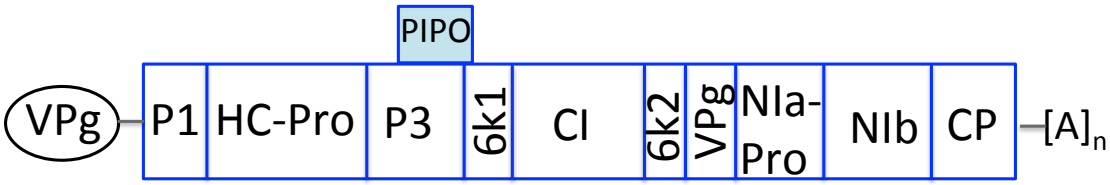


Figure 1.2 Diagram of a generalised Potyvirus genome

Potyviruses have monopartite polyadenylated positive sense RNA genomes approximately 10 kb long. The major open reading frame (ORF) is represented as a long rectangle divided by lines showing the final viral protein products. Pretty interesting ORF (PIPO ORF) is indicated as a light blue square overlapping the P3 region. A virus protein genome linked (VPg) molecule is covalently attached to the 5' terminus of the viral RNA.

1.5 Aphids

Aphids belong to the superfamily of insects Aphidoidea (order Hemiptera) that are specialist phloem feeders (Ng and Falk, 2006). Aphids are considered major pests because these insects cause losses in crop yield directly by feeding or by transmitting plant virus diseases. Aphids can colonize plant species of many different families and vector about 50% of all insect–transmitted viruses (Brault et al., 2010).

Aphid species vary in whether they are generalists or specialists, based on their degree of dietary specialization. Specialist aphids are limited to a few closely related plants, whereas generalist aphids can feed on species of more than one plant family. One of the best-studied aphid species is the generalist *Myzus persicae* (Sulzer), which has a worldwide distribution and is also known by various common names including peach-potato aphid (UK) and green peach aphid (US) (van Emden and Harrington, 2007). *M. persicae* feeds on a diverse range of hosts from more than 400 species in 40 different plant families including Arabidopsis and crops within the Solanaceae and Cucurbitaceae families (Blackman and Eastop, 2000; Louis and Shah, 2013).

1.5.1 The *Myzus persicae* life cycle

M. persicae has an alternating life cycle, consisting of sexual and asexual phases (Figure 1.3). Environmental factors, such as day length and temperature, provide cues that determine the development of the aphid into the next stage in its life cycle. In each stage of its life cycle, different morphs exist. Each morph is adapted to ensure aphid survival throughout the seasonal and climatic changes (van Emden et al., 1969). In severe climate conditions, aphids overwinter in the egg stage on its primary host. Towards the end of the winter, immature females emerge from the egg and undergo four moults to develop into adult parthenogenetic females (a stage known as fundatrix). Each fundatrix has a high reproduction rate and may give birth

to up to several hundreds of offspring, producing the early spring migrants, which leave the primary host in search of secondary host plants (Eastop, 1977). Parthenogenesis offers a short life cycle advantage whereby ovarian development starts at the embryo stage while embryos are still forming inside embryonic mothers. This phenomenon is called telescoping of generations (Dixon, 1992). The ability to produce parthenogenic wingless forms offers a further fitness advantage as resources can be allocated more towards reproduction, which results in a high rate of population increase (Dedryver et al., 2010).

During summer, induced by the longer day length, most aphids are parthenogenetic and wingless. Eventually, overcrowding leads to the production of winged offspring, which emigrate from the host plant in search of a new host (Eastop, 1997). Emigration favours aphid-vectored virus transmission (van Emden et al*.,* 1969). Aphid population reaches its peak during spring and late summer. Thus, these are the times of the year when most crop plants are damaged due to aphid infestation. At the beginning of autumn, shorter day length can induce the production of winged immigrants and winged males (van Emden et al*.,* 1969; Eastop, 1997). Under controlled conditions most aphids, including *M. persicae*, can be maintained indefinitely as a clone of parthenogenetically reproducing females.

1.5.2 Aphid host selection

Aphids follow olfactory and visual cues to locate their hosts (Reviewed by Nalam et al., 2018; Powell et al., 2006). To locate host plants, aphids rely on sensory information such as colour and odour to land on a leaf surface and evaluate its characteristics (Bruce et al., 2005; Powell et al., 2006). Olfactory sensory neurones located in aphid antennae detect plant volatile compounds (Bruce et al., 2005). Aphid responses to single compounds differ with plant volatile blends (Webster et al., 2008). Plant volatile blends are released by plants in specific ratios and this is critical

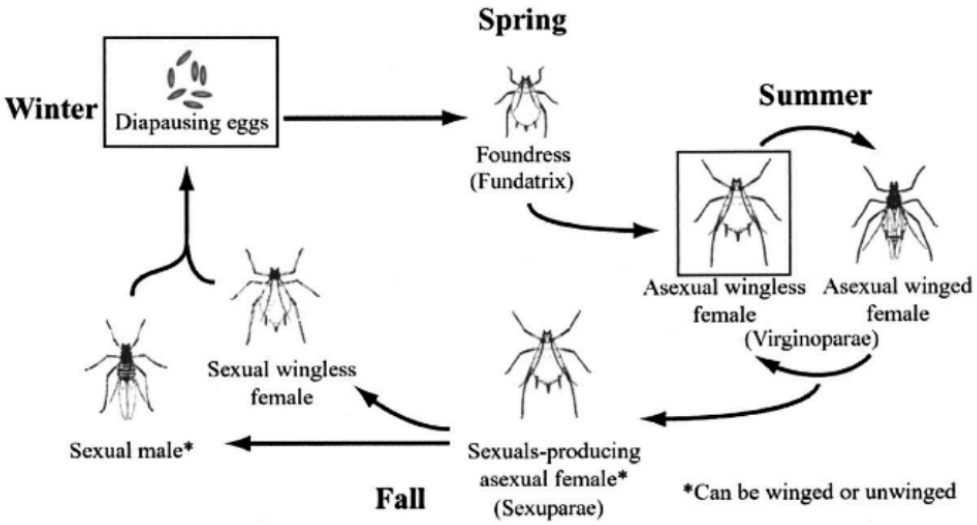


Figure 1.3 The aphid life cycle

Aphids have alternating sexual and asexual (parthenogenetic) phases within the same lineage. However, they can exhibit irreversible transitions from cyclical to obligate parthenogenesis within the same species. Illustration taken from Miura et al. (2003).

for specific plant-aphid interactions (Bruce and Pickett, 2011). For example, *Aphis fabae* is more attracted to the entire volatile blend of its host *Vicia faba* than to individual volatile compounds isolated from the blend (Webster et al., 2008). Thus, plant volatile blends play a more relevant role than individual compounds in plant-aphid interactions (Schröder et al., 2017).

Aphids further assess host suitability by probing host cells to acquire gustatory cues. The epipharyngeal gustatory organ of aphids is a chemoreceptive structure located in the food canal (Wensler and Filshie, 1969). Subsequently, sensory cells in the epipharyngeal gustatory organ make direct contact with ingested fluids passing through the food canal and permit the aphid to detect chemical stimuli after the stylet have penetrated the host cells (Wensler and Filshie, 1969). As a result, a plant is chosen as suitable for settling and reproduction only after plant contact is made (Bruce et al., 2005; Powell et al., 2006). Stylet penetration starts as brief probes to the epidermal layer (Tjallingii and Esch, 1993). Several initial probing activities are essential to establish plant suitability by aphids (Prado and Tjallingii, 2007). Plant rejection can occur at any stage of host evaluation process (Powell et al., 2006). The evaluation of plant cells continue with longer probes into the mesophyll and parenchyma tissues, and culminates if the host is acceptable by feeding from the phloem sieve elements (Tjallingii and Esch, 1993).

1.6 Plant defences against viruses

1.6.1 RNA silencing

RNA silencing can provide a primary defence against viruses (reviewed by Carr et al., 2010). RNA silencing, also known as RNA interference (RNAi), is a mechanism that protects cells against “invading” nucleic acids (e.g. viruses, retrotransposons), and regulates the expression of certain genes during development and in response to abiotic and biotic stresses. RNA silencing is triggered by double stranded RNA (dsRNA). After recognition, dsRNA-specific cleaving enzymes known as Dicers in animals or Dicer-like proteins (DCL) in plants cleave the dsRNA into short 21-24 nucleotide small- interfering RNAs (siRNAs) (Baulcombe, 2004). The siRNAs are incorporated into RNA-induced silencing complexes (RISC) containing Argonaute (AGO) proteins. A RISC is an effector complex in which AGO proteins degrade the target RNA in a sequence-specific manner guided by siRNAs (Burgyán and Havelda, 2011). The main steps involved in RNA silencing are shown in Figure 1.4.

MicroRNAs (miRNAs) are endogenous non-coding RNAs of 18-25 nucleotides in length that negatively regulate their complementary mRNAs at the posttranscriptional or at the translational level (Bartel, 2004). The miRNA pathway is similar to the siRNA pathway but they differ in their biogenesis (Baulcombe, 2005). In plants, mature miRNAs are generated from the premiRNA transcript by DCL1 (RNase III-like ribonucleases) in a sequence specific manner. Mature miRNAs are loaded into RISC complexes to degrade mRNAs or inhibit their translation (Bartel, 2004).

Many plant viruses counteract silencing-mediated resistance by producing VSRs (viral suppressor of RNA silencing) (Palukaitis and Carr, 2008). The CMV 2b protein is a VSR that inhibits silencing by binding small RNAs and interacting with AGO1 and AGO4 to prevent RISC assembly

(Goto et al., 2007; González et al., 2010, 2012; Kanazawa et al., 2011; Duan et al., 2012). The 2b protein also inhibits AGO1-mediated miRNA cleavage of host mRNAs (Zhang et al., 2006) and AGO4-mediated transcriptional silencing (Hamera et al., 2012).

1.6.2 Natural genetic resistance to viruses

There are several forms of genetic resistance to plant viruses but the best studied are controlled by single dominant or recessive genes. Plants have an innate immune system that counteracts invasion by microbes, including potential pathogens. The outcome of an attempted infection is ultimately dependent on the host plant and pathogen genotype (Moffett, 2009, 2017). The first line of defence is provided by non-host resistance in which receptors, pattern recognition receptors (PRRs) for "generic" microbial factors PAMPS (pathogen associated molecular patterns) trigger localised defences that are sufficient to ward off most microbes (Zipfel, 2014). These PRRs recognize conserved structures in pathogens such as flagellin from the flagella of bacteria or chitin from fungal cell walls to induce PAMP triggered immunity (PTI) response (Jones and Dangl, 2006; Jones et al., 2016).

Certain pathogens termed "virulent" can overcome these basal defences. Such virulent pathogens can be rendered "avirulent" by resistance (R) proteins, which are encoded by corresponding *R* genes. The specificity of resistance is based on the relationship between the *R* gene and a corresponding dominant *Avirulence* (*Avr)* gene in the pathogen. Many *R* genes encode proteins containing nucleotide-binding and leucine-rich repeat domains (NLR proteins) (Jones and Dangl, 2006; Jones et al., 2016). NLRs proteins recognize pathogen encoded protein "effector" delivered into the host cells (most plant pathogens) or synthesized in the host cell (viral proteins) (Moffett, 2017).

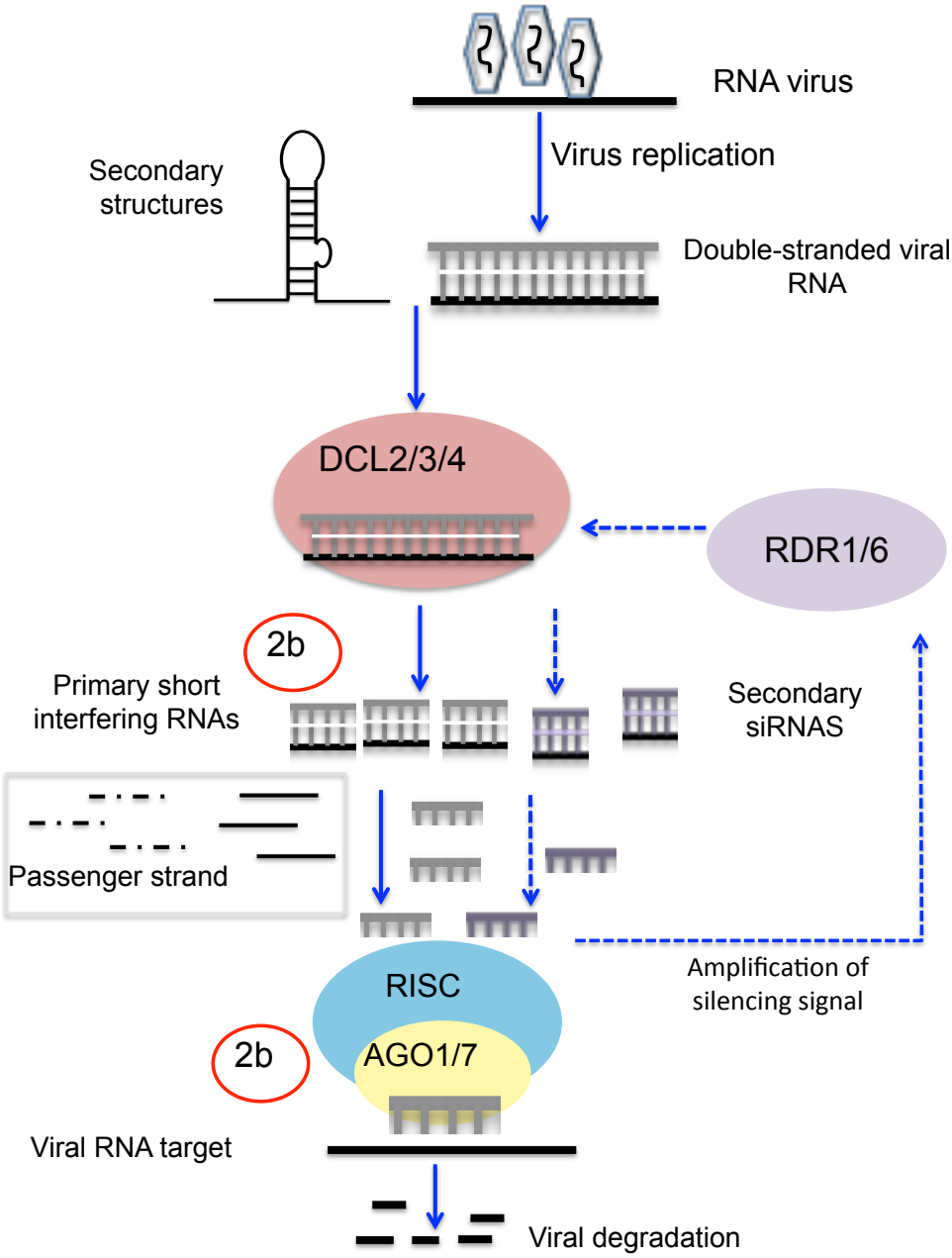


Figure 1.4 Mains steps involved in viral RNA silencing

Double stranded (ds) regions within viral RNA molecules are cleaved by DCL ribonucleases to produce primary small-interfering (si)RNA duplexes. A passenger strand is degraded and the primary siRNA is loaded to the RISC complex. The host-encoded RNA-dependent RNA polymerases (RDRs) (RDR1 or RDR6) generate further dsRNA to produce secondary siRNA duplexes. These siRNAs may then direct additional AGO-mediated cleavage against viral-derived RNAs. The 2b VSR (viral silencing suppressor) is shown in red circles and host-encoded proteins in coloured ellipses.

Arabidopsis has naturally occurring resistance genes against plant viruses. For example, *RCY1* confers hypersensitive response (HR) resistance to CMV strain Y in the Arabidopsis accession C24. *RCY1* has been transferred to Arabidopsis accession Col-0, which is susceptible to CMV strain Y (Sekine et al., 2008). In transgenic Col-0 Arabidopsis expressing *RCY1* the systemic spread of CMV was inhibited (Sekine et al., 2008). The HR is sometimes accompanied by programmed cell death in host tissue but this is not necessarily required for pathogen restriction (reviewed by Carr et al., 2010). The HR is accompanied by changes in the level of salicylic acid (SA), jasmonic acid (JA), nitride oxide (NO), ethylene, and reactive oxygen species (ROS) (Carr et al., 2010).

Viruses recruit plant factors to aid their infection cycle. Absence of these factors or the presence of mutant or aberrant versions may confer recessive (r) resistance to viruses. In contrast to dominant resistance, recessive resistance is thought to be more durable (Lecoq et al., 2004). For example, in Arabidopsis a mutation in the *CUM1* gene reduces expression of eIF4E. Since eIF4E is needed by the virus, virus accumulation and cell-to-cell movement of CMV are inhibited in the mutant plant (Yoshii et al., 1998, 2004). Interestingly, accumulation of the 3a movement protein was strongly reduced in *cum1* mutant protoplasts, whereas RNA 3 accumulation was normal. Thus, the mutation affects either the translation of CMV RNA3 or the stability of the 3a protein. Comparison of CMV RNAs 3 and 4 sequences showed that their identical 3'-untranslated regions (UTR) contained translation-enhancing elements, which might enhance protein expression (Yoshii et al., 2004). Potyvirus-inhibiting *r* genes are well studied. Recessive resistance mediated by eIF4E-type factors was studied in Arabidopsis mutants that exhibited decreased susceptibility to the potyviruses, tobacco etch virus and TuMV (Whitham et al., 1999; Lellis et al., 2002). The specific interaction between potyviral VPgs (Section 1.4) and translation initiation

factors explains the basis for recessive resistance against potyviruses. The VPg of TuMV interacts with eIF(iso)4E in Arabidopsis (Wittmann et al., 1997) and the knockout of both *eIF(iso)4E* allelles conferred resistance to TuMV in Arabidopsis (Sato et al., 2005). Thus, mutations in plant genes encoding factors necessary for viral infection can engender virus resistance (see Section 1.7.2).

Resistance genes against viruses are more durable than resistance genes against fungal or oomocyte even though viruses have a high rate of mutation (García-Arenal and McDonald, 2003). It is estimated that those mutations may have a pleiotropic effect determined by type of mutation and susceptibility of the host (Moreno-Pérez et al., 2016).

1.7 Transgenic resistance to viruses

1.7.1 Early work: Pathogen-derived resistance

In the work described in this thesis, virus-resistant transgenic plants were used in some experiments to simulate the effect of including resistant plants in field designs. The ability to engineer resistance in plants was first demonstrated in 1986 when Roger Beachy's group generated transgenic tobacco plants expressing the *CP* gene of tobacco mosaic virus (TMV). TMV-induced disease was delayed in these plants (Powell-Abel et al., 1986). Resistance resulted from the effects of the transgenically expressed CP on uncoating of virus particles and inhibition of systemic movement (Nelson et al., 1987; Register and Beachy, 1988). This was the first working example of 'pathogen-derived' resistance, i.e. where transgenic expression of virus-derived sequences renders a plant resistant to infection by the virus from which the expressed sequence was derived. Since then, many virus-resistant plant lines have been developed using the pathogen-derived resistance approach (reviewed by Cillo and Palukaitis, 2014). Examples of pathogen-derived resistance include the use of

gene sequences encoding replicase components, movement proteins, and viral proteases, as well as CP (Palukaitis and Zaitlin, 1997). However, early attempts to engineer resistance against potyviruses indicated that there was not always a relationship between protein level and resistance (Lindbo et al., 1993). Further studies led eventually to the realization that pathogen-derived resistance could in certain cases result from the action of the expressed protein, the RNA, or both (Cillo and Palukaitis, 2014). The realization that the expressed RNA by itself could create resistance led directly to the discovery of RNA silencing (Palukaitis, 2011). Subsequently, and until the recent development of gene editing techniques, most efforts to genetically engineer virus resistance have been based on RNA silencing.

1.7.2 Targeted approaches: RNA silencing mediated resistance and gene editing

RNA silencing-mediated viral resistance via transgenic expression of viral-derived sequences as anti-sense, inverted repeat, hairpin (hp), or artificial microRNA (amiRNA) has been achieved in many plant species (reviewed by Cillo and Palukaitis, 2014; Galvez et al., 2014). As described in Section 1.6.1, miRNAs are small non-coding endogenous RNAs that regulate gene expression at the post-transcriptional level in a sequence specific manner. MiRNAs in plants are produced from endogenous single stranded primary miRNA non coding transcripts with wide fold-back structures, that are processed and generated by DCL and AGO proteins (Baulcombe, 2005). Many studies have established that the alteration or mutation within the mature miRNA sequence has no effect on its biogenesis (Vaucheret et al., 2004). This led to the development of artificial microRNA (amiRNA), where a modified miRNA confers virus resistance in Arabidopsis by stable expression of amiRNAs targeting RNA sequences that encode viral proteins (Niu et al., 2006). For example, transgenic Arabidopsis plants expressing amiRNAs targeting the viral gene

segments encoding the silencing suppressor P69 of turnip yellow mosaic virus (TYMV) and HC-Pro of TuMV are resistant to TYMV and TuMV, respectively (Niu et al., 2006). Furthermore, amiRNAs have been expressed in Arabidopsis to confer resistance to CMV by targeting the conserved 3'UTR sequence of CMV genomic RNAs (Duan et al., 2008). The technique has also been successfully used to develop tomato lines resistant to CMV (Zhang et al., 2011).

RNA silencing can be induced in transgenic plants by insertion and expression of constructs that are composed of inverted repeats of viral sequences separated by an intron spacer (hairpin construct:hp). The construct gives rise to dsRNA transcripts that trigger RNA silencing (Waterhouse et al., 1998; Smith et al., 2000) (Section 1.6.1). DCLs process the expressed hpRNAs in transgenic plants into siRNAs which will bind to entering viral RNA sequences when plants are challenged with the virus. In plants, hpRNAs are processed into siRNAs by the same enzymes involved in antiviral RNA silencing (Fusaro et al., 2006). Resistance to viruses in plants expressing hpRNA constructs has been reported in Arabidopsis, *N. benthamiana*, tomato, potato, and cassava (Mitter and Dietzgen, 2012).

Recently, genome editing based on CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas9 (CRISPR-associated protein 9) was used to generate virus-resistant plants. For example, *eIF4(iso) 4E* was edited in Arabidopsis to confer resitance to TuMV (Pyott et al., 2016) and in cucumber plants the *eIF4E* gene was mutated, rendering the plants resistant to three potyviruses (Chandrasekaran et al., 2016). The latter shows that gene editing can be used to develop broad resistance. Although with gene editing it is necessary to introduce foreign DNA into the plants in order to express the Cas9 and guide RNA, these constructs are subsequently removed from the line by back-crossing and so the resulting mutant plants are not transgenic.

Alternatives to virus-resistant transgenic plants include also the delivery of dsRNA or siRNAS via topical application to induce RNA silencing against viruses. Spray applications of dsRNA using nanoparticles as delivery systems that support the stability and delivery of dsRNA onto the leaf surfaces have been recently reported (Mitter et al., 2017). Systemic movement of the dsRNA was observed and tobacco was protected against pepper mild mottle virus and CMV for up to 20 days, which is a great improvement compared with naked dsRNA that induces protection for only up to 5 days (Mitter et al., 2017).

DsRNA targeting RNA encoding proteins involved in aphid transmission is another method to inhibit virus transmission. The aphid protein Ephrin receptor (EpH) has been identified as a membrane-bound receptor involved in turnip yellows virus (TuYV) acquisition and transmission (Mulot et al., 2018). Aphids treated with dsRNA against the Eph protein were not able to internalize TuYV inhibiting transmission of the virus (Mulot et al., 2018).

1.8 Plant defences against aphids

1.8.1 Plant secondary metabolites against aphids

Plants use secondary metabolites to defend against herbivorous insects including aphids (Kessler and Baldwin, 2002). For example, trichomes, which are physical barriers to insect movement, also provide chemical defences. For instance, glandular trichomes in the wild potato *Solanum berthaultii* release the aphid alarm pheromone, (E)-β-farnesene, which promotes aphid dispersal (Gibson and Pickett, 1983). Some plant defences against aphids can be classified as antixenosis and antibiosis. Antixenotic responses influence aphid behaviour in terms of plant choice and feeding

behaviour. In contrast, antibiosis influence aphid physiology by inhibition of growth, reproduction and/or survival (Nalam et al., 2018).

Plants synthesise a variety of secondary metabolites that act as defence compounds against pathogens and herbivores (de Vos and Jander, 2009). Glucosinolates, which are an example of antixenotic defence, are plant secondary metabolites produced in the Brassicaceae family. Glucosinolates are amino acid-derived thioglycosides. Methionine, tryptophan and phenylalanine undergo a number of reactions to produce aliphatic, indole or benzenic glucosinolates, respectively (Kliebenstein et al., 2001b). Upon cell damage caused by mechanical or feeding wounding, glucosinolates are hydrolysed by myrosinases to produce toxic compounds such as, isothicyanates, thiocyanates and nitriles (de Vos et al., 2007; Halkier and Gershenzon, 2006) that have defensive properties against insect herbivores (Burow et al., 2009). Brassicaceae specialist herbivores overcome glucosinolate-based defences and exploit them as oviposition and feeding cues (Miles et al., 2005). However, these toxic compounds act as feeding deterrents for generalist insects. For example, the indole glucosinolate, 4-methoxy-indol3yl-methylglucosinolate (4MI3M), is a feeding deterrent for the generalist herbivore *M. persicae* (Kim and Jander, 2007; Kim et al., 2008).

Arabidopsis accessions display natural qualitative and quantitative variation in glucosinolate content. Variation in glucosinolate profiles among Arabidopsis accessions has been used to study glucosinolate interactions with insect herbivore attack (Kliebenstein, 2004). For example, Arabidopsis lines with higher glucosinolate levels were more resistant to attack by the generalist herbivores *Trichoplusia ni* and *Spodoptera exigua* (Kroymann et al., 2003). Natural variation in Arabidopsis accessions conditions specific interactions with generalist and specialist aphids (Kuśnierczyk et al., 2007). Although aphids cause less feeding damage than chewing

insects, aphid feeding still induces plant defences (Nalam et al., 2018). Infestation of Arabidopsis plants by the generalist aphid *M. persicae* or the specialist aphid *Brevicoryne brassicae* induces expression of indole glucosinolate biosynthesis genes. *M. persicae* infestation induces the conversion of indol-3-ylmethyl glucosinolate (I3M) to the aphid-deterrent compound (4MI3M) (Kim and Jander, 2007).

1.8.2 Aphid resistance genes

Aphid-specific *R* genes have been isolated and characterized but the number of genes cloned is limited (Nalam et al., 2018). The *Mi* gene of tomato plants confers resistance to the potato aphid (*Macrosiphum euphorbiae)*, the whitefly (*Bemisia tabaci)* and root-knot nematodes (*Meloidogyne* spp.) (Rossi et al., 1998; Milligan et al., 1998; Nombela et al., 2003). The *Vat* gene of melon confers resistance to *Aphis gossypii* colonization and decreases the transmission of plant viruses by this aphid (Dogimont et al., 2014). However, when other aphid species transmit viruses to melon, *Vat* does not prevent virus infection (Boissot et al., 2016). *Mi* and *Vat* condition HR-type responses against aphids (Kanvil et al., 2014). Kanvil and colleagues (2014) evaluated aphid resistance on twenty-three *Medicago truncatula* genotypes against eight *Acrythosiphon pisum* clones. They found that aphid virulence and host resistance are highly dependent on genotype of both aphid and host. Thus, *R* gene mediated plant defence against aphids is determined by host gentoype and aphid biotypes.

1.9 Plant viruses induce changes in host-vector interactions

It has been suggested that non-persistent transmission occurs most efficiently when viruliferous aphids disperse quickly from infected hosts to new host plants (Powell, 2005; Brault et al., 2010; Mauck et al., 2012; Westwood et al., 2013a). How does a virus that does not replicate in its vector promote its own transmission? There is evidence that virus infection causes changes in plant biochemistry that influences

vector behaviour and performance (Ingwell et al., 2012; Mauck et al., 2014; Carr et al., 2018). *M. persicae* performance was evaluated in potato plants infected with three different types of viruses (Castle and Berger, 1993). The types of viruses were chosen based on their mode of transmission as follows: PLRV a circulative virus dependent on *M. persicae* for transmission, PVY a non-circulative virus also dependent on *M. persicae* for transmission and PVX a virus non transmissible by *M. persicae*. Castle and Berger (1993) reported that infection of potatoes by PLRV or PVY enhanced vector performance but PVX infection did not improve aphid performance compared with the virus-free potato plants. The results suggested that viruses that are aphid transmissible might induce changes in host quality that alter aphid performance. In contrast, the pea aphid *A. pisum* on *V. faba* plants infected with pea enation mosaic virus (a circulative-persistent virus), bean yellow mosaic virus (a non-persistent virus) or bean mottle virus (a non-aphid transmissible virus), did not exhibit an improvement in aphid performance (Hodge and Powell, 2008). However, aphids preferred to settled on the three types of virus infected plants studied (Hodge and Powell, 2008). Thus, it was concluded that virus infection might not improve the already high quality host-aphid interactions between *A. pisum* and *V. faba* and aphid preference to settle on virus infected plants was not correlated with aphid performance (Hodge and Powell, 2008). The virus-vector-host interactions may also depend on vector transmission efficiency. Chesnais and colleagues (2019) studied the effects of infection of *Camelina sativa* with cauliflower mosaic virus (a semi-persistent and non circulative virus) and turnip yellows virus (a persistent and circulative virus) on vector performance of two aphid species. The authors studied the effects of virus infection on the generalist *M. persicae* that is an efficient vector of both cauliflower mosaic virus and turnip yellows virus; and the Brassicaceae specialist *Brevicoryne brassicae* which is a poor vector of turnip yellows virus and efficient vector of cauliflower mosaic virus. Chesnais and colleagues (2019) found that virus infection with the persistent and circulative virus turnip yellows virus

improves aphid performance of *M. persicae* but the effects were neutral for the specialist *B. brassicae*. In contrast the authors reported that aphid performance of *M. persicae* and *B.* brassicae was negative when *C. sativa* plants were infected with the semipersistent noncirculative cauliflower mosaic virus. The results of that study suggested, that virus infection induces changes on vector performance in a aphid species specific manner but it is also dependent on virus transmission efficiency of the vector (Chesnais et al., 2019). Thus, viruses can have indirect effects on their vectors by changing the properties of the host.

1.9.1 Plant virus-induced changes in plant volatiles

Viral manipulation of aphid behaviour has been observed in viruses with persistent and non-persistent transmission. For example, the aphid *Rhopalosiphum padi,* after acquring the persistently transmitted barley yellow dwarf virus (BYDV) from *in vitro* feeding, preferred to settle on non-infected wheat plants, while non-viruliferous aphids also fed *in vitro* preferred to settle on BYDV-infected plants (Ingwell et al., 2012). Similarly, viruliferous (i.e carrying potato leafroll virus (PLRV)) *M. persicae* aphids preferred to settle on healthy potato plants than PLRV-infected potato plants (Rajabaskar et al., 2014), but non-virulifeorus aphids settled preferentially on PLRV-infected potato plants than healthy plants . Both BYDV and PLRV are viruses that do not replicate in the vector and require high virus accumulation in the plant and longer vector feeding to be acquired and inoculated. The results showed that host selection behaviour of the aphid changed after virus acquisition (Ingwell et al., 2012). In both studies, it was determined that aphid discrimination between virus-infected and non-infected plants was mediated by VOC (volatile organic compound) emission whereby in Y-tube assays aphids preferred trapped headspace VOCs from BYDV- and PLRV-infected plants. Thus, it was proposed that the preference of viruliferous aphids for non-infected host plants enhances virus acquisition and promotes virus transmission (Ingwell et al., 2012; Rajabaskar et al., 2014).

1.9.2 Viral proteins and host-vector interactions

Studies dissecting the involvement of viral proteins in host-vector interactions have shown that viral proteins might mediate host-vector interactions. The CMV 2b protein as discussed before is a multifunctional protein (Section 1.3.2). The 2b protein interferes with host gene expression and defence signalling governed by several signals including salicylic acid (Ji and Ding, 2001; Zhou et al., 2014) and jasmonic acid (Lewsey et al., 2010). These two defensive signals are most important, respectively, for systemic acquired resistance against pathogens, and defence against insects and certain necrotrophic pathogens (Palukaitis and Carr, 2008). Constitutive expression of Fny-CMV 2b protein in transgenic Arabidopsis inhibited methyl-JA induced changes in expression of 90% of JA-regulated genes (Lewsey et al. 2010). Similarly, in *N. benthamiana* the expression of a Subgroup II CMV 2b protein (Kin-CMV) also inhibited the responses to JA (Westwood et al., 2014).

Tobacco plants infected with a CMV mutant lacking the 2b protein (CMVΔ2b) are more resistant to *M. persicae* (Ziebell et al., 2011). However, aphid survival and growth was improved on plants infected with wild-type CMV. This suggests that the 2b protein aids vector survival on CMV-infected tobacco by inhibiting virus-triggered aphid resistance (Ziebell et al., 2011). In Arabidopsis, Fny-CMV infection had a different effect on the interaction between the host and *M. persicae*, as virus infection induced aphid resistance (Westwood et al., 2013a). In this host the 2a protein stimulates the accumulation of 4MI3M by triggering PTI (Westwood et al., 2013a) (Figure 1.5), discouraging aphids settling on Fny-CMV infected Arabidopsis accession Col-0 plants and enhancing aphid movement to new hosts which is thought likely to enhance transmission of CMV (Westwood et al., 2013a).

In other viruses, for example, TuMV infection in Arabidopsis alters ethylene signaling to reduce host resistance to aphids thereby enhancing reproduction (Casteel et al., 2014, 2015; Bak et al., 2017). NIaPro relocalises to the vacuole in the presence of *M. persicae* in Arabidopsis and *N. benthamiana* but not in tobacco plants (Bak et al., 2017). Thus, the study of viral proteins and how they affect host-vector interactions shows that viral proteins may alter signalling responses to induce changes in vector behaviour and performance.

1.10 Aphid and virus management

Developing virus-resistant varieties is a lengthy process, especially by traditional breeding since introgressing resistance genes into crop cultivars from wild relatives is complex. Insecticides are poorly effective for controlling non-persistently transmitted viruses. For example, an insecticide (imidacloprid) was evaluated for its ability to reduce spread of PVY and PLRV. However, only a reduction of PLRV spread (a persistently transmitted virus) was observed, but no effect on the spread of the non-persistently transmitted PVY was found (Boiteau and Singh, 1999). Thus, viruses with non-persistent transmission are difficult to control with insecticides because these chemicals do not act quickly enough to kill the insects during brief acquisition and inoculation probes. Paradoxically, there is a report of an increase of virus incidence in crops when insecticides are applied because these chemicals increase probing activity and movement of aphids (Roberts et al., 1993). Furthermore, the use of pesticides is financially impractical for smallholder farmers in developing countries (Worrall et al., 2015) and there is an increasing threat of pesticide-resistant strains (Jones, 2014a).

Alternatives to insecticides focus on manipulation of pest behaviour and the use of trap cropping, which is a strategy of integrated pest management (Pickett et al., 2014). Trap crops are defined as plants that attract pests to protect the target crop

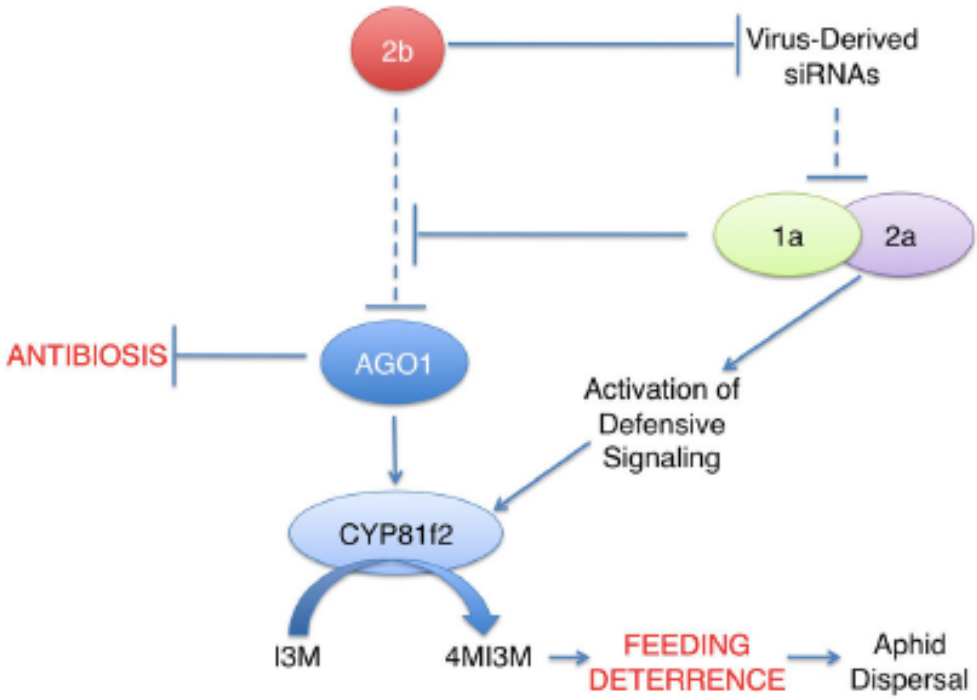


Figure 1.5 Induction of aphid feeding deterrence during CMV infection

The 2b RNA silencing suppressor protein of CMV inhibits antiviral silencing through binding of virus-derived siRNAs, allowing the 1a and 2a protein to accumulate. The 2b protein can also bind to and inhibit AGO1, which positively regulates the expression of the gene for CYP81F2, which catalyses formation of the aphid feeding deterrent compound 4-methoxy-indo3yl-methylglucosinolate (4MI3M) from its precursor indol-3-yl-methylglucosinolate (I3M). The multifunctional 2a protein (RNA-dependent RNA polymerase) induces PAMP-triggered immunity causing increased 4MI3M production. This results in aphid feeding deterrence (antixenosis), which is thought likely to increase aphid dispersal and thus enhance transmission of a non-persistently transmitted virus like CMV. If 2b is not regulated by 1a protein (a multifunctional protein that is also a replicase component), inhibition of AGO1 results in induction of antibiosis against aphids, which will not favour aphid-mediated virus transmission. In the illustration, the truncated lines indicate inhibition and the truncated dashed-lines indicate down-regulation. Taken from Westwood and colleagues (2013a).

from pest attack. A trap plant that acts as a “sink” for the non-persistent plant viruses has been evaluated by using wheat (*Triticum aestivum*) as a trap crop and muskmelon (*Cucumis melo)* as the main crop (Toba et al., 1977). This virus management strategy is suggested to control winged aphids, which are considered the major source of primary virus infection in a crop (i.e introduction of a virus into a field from a source outside of the field). However, colonising aphid species and in particular wingless aphids are also important for secondary spread as they crawl to adjacent plants thereby amplifying the spread of the virus (Raccah et al., 1985). Secondary spread is defined as the introduction of a virus from source plants within a field and it may depend on vector population and behaviour as well as number and state of infection of source plants (Kennedy, 1976). Toba and colleagues (1977) suggested the inclusion of non-crop plants to provide additional feeding sites for viruliferous aphids. Currently the use of semiochemicals, which are volatiles that attract or repel insects, have led to the development of “push-pull” systems (Pickett and Khan, 2016). In the “push-pull” system, the main crop is protected by companion crops, which “push” or reduce pest colonisation and “pull” or attract and trap the pest. Thus, pest management is achieved by exploitation of the semiochemistry of the companion crops.

Management of insect transmitted plant diseases could potentially be achieved by inhibition of virus transmission (Westwood and Stevens, 2010; Groen et al., 2017). Manipulating olfactory cues repels insects under controlled conditions (Beale et al., 2006). Transgenic wheat plants constitutively emitting an alarm pheromone, (E)-β-farnesene, repelled aphids and attracted parasitic wasps in tests under controlled conditions. However, under field conditions, the plants did not repel aphids, indicating more work is needed to make this promising approach robust enough for field use (Beale et al., 2006; Bruce et al., 2015). In my thesis work, I explored the question:

Can an additive effect of aphid olfactory stimuli and virus-resistant plants be used to inhibit virus spread?

1.11 Working model and objectives of study

1.11.1 Working model and hypotheses

For my project I used a model system extensively used in our lab, the CMV, Arabidopsis and *M. persicae* pathosystem. Previous work in the group suggested the hypothesis that plants infected with non-persistently transmitted viruses exhibit two types of responses in vector-host interactions (Westwood et al., 2013a; Ziebell et al., 2011).

Type 1 hosts: CMV-infected Arabidopsis accession Col-0 plants are examples of Type 1 hosts (Westwood et al., 2013a). In this type of host, CMV induces feeding deterrence, which was proposed to enhance increased CMV transmission (Westwood et al., 2013a; Mauck, 2016). The hypothesis for type 1 hosts is that virus infection with non-persistent viruses induces changes in plant biochemistry that causes negative changes in aphid behaviour and performance that encourage viruliferous aphid movement away from the virus infected plant which will enhance virus spread to new host.

Type 2 hosts: Aphids may remain on CMV-infected or TuMV-infected plants of some species, as they are more palatable or exhibit less resistance to aphids (Casteel 2014; Ziebell et al., 2011). Thus, the hypothesis for Type 2 host is that virus infection encourages aphid settling and reproduction on the host and as a result virus spread to new hosts is reduced. This strategy may benefit the virus by promoting aphid population growth. This is generally thought to inhibit spread of non-persistenly transmitted viruses (Ingwell et al, 2012; Mauck, 2016). However, type 2 hosts have

been proposed to be refuges for the virus and its vector during times of drought, cold or other stress (Ziebell et al., 2011). More recently, our group have proposed, based on epidemiological modelling, that the increased population density of Type 2 plants might encourage birth of winged aphids, which would drive long-distance dissemination of viruses (Donnelly et al., in press). Ziebell and colleagues (2011) showed that CMV-infected tobacco are Type 2 plants. In this thesis I showed that CMV-infected Arabidopsis accession Ei-2 plants may have some of the characteristics of Type 2 plants (Chapter 4). A similar effect has been found in Arabidopsis plants infected with TuMV (Casteel et al., 2014). In particular, Casteel and colleagues (2014) found that aphids grew and reproduced better when placed on virus-infected plants. However, data presented in this thesis appears to contradict this (Chapter 4).

1.11.2 Objectives

The main objective of my project was to test whether under controlled conditions I could use the knowledge gained from previous work to manipulate virus-host-vector to inhibit virus transmission (Section 1.9). My investigations tie in with epidemiological modelling and a longer project involving field experiments in East Africa to test approaches to disrupt virus spread in crops such as common bean. My main activities are listed below.

1. I generated independent transgenic lines resistant to CMV and TuMV (Chapter 3). I used Arabidopsis accessions Col-0 and Ei-2 as the plant backgrounds to express constructs that confer virus resistance by RNA silencing (Section 1.6.1). I assessed the level of virus resistance by mechanical and aphid-inoculation: using mechanical and aphid-mediated inoculation I identified highly and fully resistant plants to CMV and TuMV. I evaluated whether these virus-resistant plants exhibited any effect on aphid behaviour that might influence my experiments to manipulate aphid behaviour

and virus transmission (Chapter 5). Additionally, I generated *N. benthamiana* plants expressing a construct complementary to the conserved *P3N-PIPO* transcriptional slippage site (Section 1.4) to test whether this would confer resistance to a broad range of potyviruses.

2. I investigated the "Type 1 and Type 2" hypotheses (Westwood et al., 2013a) using different Arabidopsis accessions (Chapter 4). It was previously found that not all Arabidopsis accessions exhibit the same changes as Col-0 on host-vector interactions following CMV infection (Groen et al., unpublished). I further investigated whether CMV infection induced different responses in Arabidopsis accessions by looking at additional aphid behaviour and performance assays. In addition, I investigated whether CMV infection affected the emission of VOCs by Arabidopsis in two accessions and how this may affect aphid host location and settling. I also investigated whether Arabidopsis accessions exhibited different levels of attractiveness to aphids. I aimed to identify an Arabidopsis accession that was more attractive to aphids than the Col-0 Arabidopsis accession previously used in experiments.

3. I used microcosm experiments under controlled growth conditions to explore the use of plant mixtures to manipulate aphid behaviour and to inhibit virus transmission (Chapter 5). I assessed whether CMV infection induces emigration of viruliferous aphids to neighbouring plants. I also aimed to demonstrate that mixtures of two Arabidopsis accessions that exhibit different level of attractiveness to aphids (Chapter 4) could be used to manipulate aphid behaviour. In addition, I aimed to test whether incorporating small proportions of resistant plants randomly distributed into mixtures could diminish virus spread. Finally, I assessed whether including virus-resistant

plants that are also attractive to aphids could further improve the inhibition of virus spread.

Chapter 2. Materials and Methods

2.1 Reagents and Non-biological materials

2.1.1 Chemicals and molecular biology reagents

Chemicals were obtained from Sigma-Aldrich (Gillingham, UK), Fisher Scientific (Loughborough, UK), New England Biolabs (Ipswich, Suffolk, UK), Promega (Southampton, Hampshire, UK), Invitrogen (Paisley, Renfrewshire, UK), Bioline Reagents Ltd (London, UK) and Ambion (Austin, Texas, USA). Other particular suppliers of specific chemicals, molecular biology reagents or equipment are noted in the text.

2.1.2 Sterilization of solutions and equipment

All glass flasks, bottles and plastic equipment were sterilized by autoclaving for 15 minutes at 121^{o}C at 15 pounds per square inch pressure. Other glassware, ceramics, and metal were soaked in 3.0% (w/v) sodium hypochlorite, for a minimum of 1 hour, washed in distilled water, and baked for 2 hours at 180 ^{o}C. All solutions and media were prepared using deionised, ultra-pure "Milli-Q" water (Millipore, Billerica, MA, USA), and sterilised by autoclaving, or filter sterilization for heat labile solutions (0.2 µm filter, Schleicher & Schuell, Dassel, Germany).

2.2 Biological material and growth conditions

2.2.1 *Brassica rapa*

Brassica rapa L. var. *pekinensis* (common name Chinese cabbage) was used to maintain aphid colonies used for experiments with Arabidopsis plants (see Section 2.6). Chinese cabbage seeds were germinated and plants were grown on Levington M3 compost (Fisons Plc., Ipswich, UK). The plants were kept under controlled growth room conditions at 22^{o}C with 16-hour photoperiod, and 200 µE.m^{-2}.s^{-1} light intensity

(Conviron, Manitoba, Canada). The plants were covered with micro-perforated plastic bags (Associated Packaging, Kent, UK) and secured with rubber bands at the base of the growing pots to contain the aphids.

2.2.2 *Nicotiana benthamiana* Domin

Plants of *Nicotiana benthamiana* Domin were used to propagate and bulk up CMV and TuMV (see Sections 2.2.10 and 2.2.11, respectively). *N. benthamiana,* the common laboratory accession Domin, which is highly susceptible to virus infection, was used (Wylie et al., 2015). Seeds were sown in 100 mm diameter plastic saucers and after germination at the 2-3 true leaf stage transferred to 9 cm diameter individual pots containing 4:1 M3 compost:sand mixture.

The plants were kept in growth chambers (Conviron Ltd., Winnipeg, Manitoba, Canada) and maintained under 12 hours of light (200 $\mu E.m^{-2}.s^{-1}$) at 22^{o}C, and 60% humidity. After three weeks of growth, plants were inoculated with viruses by agroinoculation (see Section 2.2.8). *N. benthamiana* was also used for the generation of transgenic potyvirus-resistant plants (Section 2.5).

2.2.3 Tobacco

Plants of *Nicotiana tabacum* L. cv. Xanthi-nc were used to maintain colonies of aphids used for experiments with *N. benthamiana* (see Section 2.6). The plants were grown under the same conditions as those used for *N. benthamiana.*

2.2.4 *Arabidopsis thaliana* L. Heynh.

Seeds of *Arabidopsis thaliana* (referred to in this thesis as “Arabidopsis”) accessions Columbia-0 (Col-0), Eifel-2 (Ei-2), Cape Verdes Islands (Cvi) and Landsberg erecta (Ler) were sown on low-nutrient F2 Levington compost (Fisons Plc., Ipswich, UK) in 15 cm diameter plastic saucers and stratified at 4 °C for 72 hours. After 10 days, seedlings were transferred to trays with 40 cells 50x48 mm in size (Desch Plantpak,

Mundon Maldon, UK). The plants used for aphid experiments (see Section 2.6) were grown on a 3:1 ratio of Levington F2 compost to sand. All plants used for aphid experiments were grown in growth chambers (Conviron Ltd., Winnipeg, Manitoba, Canada) under "short day" conditions (8 hour light period: and 200 $\mu E.m^{-2}.s^{-1}$) at 22 °C and 60% relative humidity. No insecticides were used. The biocontrol agent *Hypoaspis miles* (Hypoline m, Syngenta Bioline Ltd., Essex, UK) was used to control pests such as sciarid flies. Arabidopsis accessions used in this project were chosen based on their glucosinolate levels and glucosinolate biosynthetic genetic loci as shown in Table 2.1.

Table 2.1 Glucosinolate profile in Arabidopsis accessions

Accesssion	Glucosinolate biosynthetic genetic loci		Total glucosinolate concentration (μmol $gDWT^{-1}$)	
	GS-Elong	GS-AOP	Aliphatic glucosinolates	Indole glucosinolates
Cvi	C_3	alkenyl alelle	37.81	1.67
Col-0	C_3	null methylsulfinylalkyl allelle	7.7	3.83
Ei-2	C_4	alkenyl alelle	19.84	3.35
Ler	C_4	hydroxypropyl allelle	11.01	2.88

Four Arabidopsis accessions used in this project were chosen based on their different concentrations of total aliphatic and total indole glucosinolates and type of glucosinolate biosynthesis loci. GS-Elong, controls the production of three carbon side chains (C_3) or four carbon side chains (C_4) and GS-AOP regulates the accumulation of aliphatic glucosinolate and production of alkenyl-, hydroxypropyl-, or methylsulfinylalkyl- glucosinolates. Glucosinolate profiles were taken from previous work by Kliebenstein and colleagues (2001a).

2.2.5 Mutant Arabidopsis lines

Seeds for *cyp81f2-1* and *cyp81f2-2* in Col-0 background were available in lab stocks, courtesy of Dr. Trisna Tungadi. The seeds were previously authenticated for mutant allele studies and aphid-virus work (Clay et al., 2009; Westwood et al., 2013a). Col-0 wild-type (WT) was used as the control line for experiments performed with *cyp81f2-1* and *cyp81f2-2* mutants.

2.2.6 Myzus persicae

Myzus persicae Sulzer was used for all the aphid work described in this investigation. Virus-free cultures of wingless individuals of the polyphagous *M. persicae* clone

US1L (Devonshire and Sawicki, 1979) were available in the lab. It is an insecticide susceptible clone maintained on Chinese cabbage or tobacco plants grown in individual pots at 21°C under long day conditions. The plants were covered with micro-perforated plastic bags (Associated Packaging, Kent, UK) secured with rubber bands at the base of the growing pots to contain the aphids.

2.2.7 Viruses

2.2.7.1 Cucumber mosaic virus

Cucumber mosaic virus strain Fast New York (Fny-CMV) (referred to as "CMV" throughout the text) was used in this work (Roosinck and Palukaitis, 1990). Infectious clones of RNA1 (pFny109), RNA2 (pFny209) and RNA3 (pFny309) were used to inoculate and propagate the virus in *N. benthamiana* via agroinoculation for later virion purification. The original infectious clones were made by Rizzo and Palukaitis (1990) but were adapted for use in agroinfection (Zhiyou Du, unpublished) (Section 2.2.8 and 2.2.10).

2.2.7.2 Turnip mosaic virus

A GPF(Green Fluorescent Protein)-expressing infectious clone of *Turnip mosaic virus* (UK-1 strain) was used in this project (Lellis et al., 2002). Methods for agroinoculation of *N. benthamiana* and virus propagation and virion purification were performed according to Olspert and colleagues (2015) (see Section 2.2.8 and 2.2.11).

2.2.8 Inoculation of plants with viruses via agroinoculation

N. benthamiana plants were agroinoculated after three weeks of growth at the four-leaf stage. Three days before infiltration of constructs, *A. tumefaciens* GV3101 cells containing pFNY109, pFNY209, or pFNY309 for FNY-CMV, or p35STuMVGPF for TuMV were grown on Luria Bertani agar medium with 50 µg/ml kanamycin and 10 µg/ml rifampicin for 48 hours at 28°C. A single colony of *A. tumefaciens* containing

the desired construct was used to inoculate 5 ml of Luria Bertani medium containing 50 µg/ml kanamycin and 10 µg/ml rifampicin in a 15 ml clear plastic tube and incubated for 8-12 hours at 28°C in a rotary shaker at 200 rpm. The cells were collected by centrifugation at 4,500 x *g* for 15 minutes at 4°C. The pelleted cells were resuspended in freshly made infiltration buffer [10 mM $MgCl_2$, 10mM MES pH 5.6, and 150 µM acetosyringone] for a final optical density at 600 nm (OD_{600}) of 0.5 and incubated at room temperature for at least 2 hours. Then, approximately 1 ml of culture was infiltrated into leaves of *N. benthamiana* using a disposable syringe without a needle (Schöb et al., 1997).

2.2.9 Inoculation of plants with viruses

For sap inoculation, systemically-infected *N. benthamiana* leaves were weighed and homogenised in two volumes of 0.05 M potassium phosphate buffer (pH 7.5) using a pestle and mortar. *N. benthamiana* plants were inoculated after three weeks of growth at the four true-leaf stage. The two oldest true leaves were dusted with Carborundum powder (silicon carbide, SiC), which was used to abrade the leaf during mechanical inoculation to aid virus entry. The sap was gently rubbed onto selected leaves with glove-covered fingers. After inoculation, the leaf surfaces were rinsed with distilled water to wash off remaining Carborundum powder. Inoculated plants were covered with propagator lids for at least 24 hours and kept in the growth rooms under the conditions described earlier (see Section 2.2.2).

For inoculation with virions, Arabidopsis plants were inoculated after four weeks of growth at the four true-leaf stage. The two oldest true leaves were dusted with Carborundum powder. Then, 2µl of a 100 µg/ml suspension of purified virions for CMV or 5 µg/ml for TuMV (Sections 2.2.10 and 2.2.11, respectively) were pipetted on each leaf and spread across the leaf surface with glove-covered finger. After inoculation, the leaves' surfaces were rinsed with distilled water to wash off the

remaining Carborundum powder. Virus-inoculated plants were covered with propagator lids for two days to maintain humidity. Plants were left to develop symptoms and only systemically-infected plants were used in experiments. Sterile water was used for mock inoculations. *N. benthamiana* plants were inoculated with viruses as previously described, although with a volume of 5 µl (5 µg/ml) of inoculum per leaf.

2.2.10 CMV virion purification

The CMV purification method was adapted and modified from previously reported protocols (Lot et al., 1972; Ng and Perry, 1999). All procedures were performed on ice or at 4^{o}C as much as possible.

Systemically-infected *N. benthamiana* leaves at 10 days post-inoculation (dpi) (see Section 2.2.8) leaves were harvested, weighed, and blended in a pre-chilled blender (Magimix, Farnham, UK), with ice-cold Buffer A [0.5 M sodium citrate pH 6.5, 5mM disodium EDTA, 0.5 % (v/v) thioglycolic acid], and chloroform, in a ratio of 1g plant tissue: 2ml Buffer A: 2ml chloroform. The homogenate was filtered through one layer of muslin pre-soaked with distilled water. The filtrate was centrifuged at 15,000 x *g (*Beckman JA-20 rotor) for 10 minutes at 4°C. The aqueous phase was recovered and divided equally into ultracentrifuge tubes underlayed with 5 ml of Buffer A plus 10% (w/v) sucrose. The solutions were centrifuged at 40,000 rpm (Beckman Ti 70 rotor) for 1 hour 15 minutes at 4 °C. The pellet obtained was resuspended in 3-5 ml of Buffer B [5 mM sodium borate pH 9.0, 0.5 mM disodium EDTA (ethylenediaminetetraacetic acid), 2% v/v Triton-X 100] and agitated for 10-12 hours at 4 °C to pellet debris. The following day, the virion suspension was centrifuged at 6,000 rpm (Beckman JA-20 rotor) for 10 minutes at 4°C. The supernatant was centrifuged at 40,000 rpm (Beckman Ti 70 rotor) for 1 hour 15 minutes at 4°C over a

5 mL cushion of Buffer C [5mM sodium borate, pH 9.0, 0.5 mM disodium EDTA]. The pellet was resuspended in 200 µl of Buffer C plus 0.02% sodium azide.

The concentration of the virus particles (mg/ml) was determined by measuring the absorbance at 260 nm and dividing this value by the extinction coefficient of 5 $ml.mg^{-1}.cm^{-1}$ (Francki et al., 1966). The virion suspension was stored at 4°C and remained infectious for approximately 3 months.

2.2.11 TuMV virion purification

The TuMV purification method was adapted and modified from previously reported protocols (Baratova et al., 2001; Olspert et al., 2015). All procedures were performed on ice or at 4^{o}C as much as possible. Systemically-infected *N. benthamiana* (10 dpi) (see Section 2.2.8) leaves were harvested, weighed, and blended in a pre-chilled blender (Magimix, Farnham, UK) with iced-cold 0.5 M potassium phosphate buffer (pH 7.5), containing 0.01M diethyldithiocarbamic acid, 0.005 M EDTA, and 1% (w/v) sodium sulphite (1g plant tissue: 2ml buffer). The homogenate was filtered through one layer of muslin pre-soaked with distilled water. The filtrate was centrifuged at 8,000 x *g (*Beckman JA-20 rotor) for 20 minutes at 4°C. The supernatant was stirred for 2 hours at 4°C with 1% (v/v) Triton X100, then PEG 6000 and NaCl were added to a final concentration of 5% (w/v) and 1.2% (w/v), respectively. The mixture was stirred for 10-12 hours at 4°C. The following day, the precipitate was sedimented at 8,000 x *g (*Beckman JA-20 rotor) at 4°C for 20 minutes and the pellet obtained was resuspended in 0.5 M potassium phosphate buffer (pH 7.5). The virion suspension was centrifuged at 8,000 x *g* (Beckman JA-20 rotor) for 10 minutes at 4°C. The supernatant was layered onto a 20% (w/v) sucrose cushion of 0.05 M potassium phosphate buffer (pH 7.5) and centrifuged at 40,000 rpm (Beckman Ti 70 rotor) for 2 hours 30 minutes at 4°C. The pellet was resuspended in 200 µl of 0.05 M phosphate buffer (pH 7.5). The concentration of the virus particles (mg/ml) was determined by

measuring the absorbance at 260 nm and dividing this value by the extinction coefficient of 2.8 $ml.mg^{-1}.cm^{-1}$ (Baratova et al., 2001). The virion suspension was stored at 4°C and remained infectious for approximately 3 months.

2.2.12 Confirmation of viral infection using ELISA

Viral infection in Arabidopsis and *N. benthamiana* plants inoculated with CMV or TuMV was confirmed using enzyme-linked immunosorbent assay (ELISA) kits. New non-inoculated leaves were collected for the assay and processed immediately. ELISA was performed using an ELISA kit for CMV and TuMV detection (Bioreba, AG, Reinach, Switzerland) according to the manufacturer's instructions. These ELISA kits use a double antibody sandwich method, using antibodies against viral coat protein for detection and quantification of plant viruses (Clark and Adams, 1977). Two technical replicates were performed for each sample. ELISA was carried out over three days. On day 1, the wells of ELISA microplates were coated with a virus specific antibody (Coating IgG). Coating IgG was diluted 1:1000 in coating buffer, and 200 µl was added to each well. The microplate was covered with Saran wrap (Dow Chemicals), placed in a humid box, and incubated for 10-12 hours at 4 °C. On day 2, the wells were emptied and washed 3-4 times using 1x ELISA washing buffer "Easy Wash 2000" [phosphate-buffered saline-Tween] (Bioreba), 10 g/l (w/v) in distilled water. Leaf samples were weighed and ground in ELISA extraction buffer [20 mM Tris buffer pH7.4, 137 mM NaCl, 3 mM KCl, 2% (w/v) PVP 24kD, 0.05% (v/v) Tween-20, 0.02% (w/v) NaN_3,] with 1:20 ratio. Each well was filled with 200 µl of test sample. The microplate was covered with Saran wrap, placed in a plastic box to maintain humidity, and incubated for 8-12 hours at 4 °C. On day 3, the wells were emptied and washed 3-4 times with washing buffer. The secondary antibody (Conjugated to alkaline phosphatase) was diluted 1:1000 in conjugate buffer (20 mM Tris, 137 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 2% PVP 24kD, 0.05% (v/v) Tween-20, 0.2% BSA (w/v), 0.02% (w/v) NaN_3, pH 7.4), and 200 µl was added to each well. The

microplate was covered and incubated for five hours at 30 °C. The wells were emptied and washed with the washing buffer 3-4 times. Substrate solution was prepared by dissolving *para*-nitrophenylphosphate in substrate buffer [1 M diethanolamine (pH 9.8), 0.02% (w/v) NaN_3) at 1 mg/ml and 200 µl was added to each well. The microplate was incubated for 30-120 minutes at room temperature until yellow colour development was visible and absorption at 405 nm was determined with a Titertek Multiscan PLUS MKII (Hunstville, AL, USA).

2.3 General molecular biological techniques

2.3.1 Total cellular RNA extraction from plant tissue

Total RNA was isolated from plant tissue using a “Trizol-like” extraction buffer [38% (v/v) Tris-buffered phenol, 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate, and 5% (v/v) glycerol]. Freshly-harvested plant tissue samples or tissue that had been snap frozen in liquid N_2 and stored at -80°C were ground in RNA extraction buffer (100 mg:1 ml). The homogenate was transferred into a 1.5 ml microfuge tube and placed on ice for 5 minutes for complete dissociation of nucleoprotein complexes. The samples were centrifuged at 18,000 x *g* for 10 min at 4°C in a precooled HERMLE Z-400 K bench centrifuge (HERMLE, Labortechnik, Wehingen, Germany). The supernatant was transferred to new pre-chilled microfuge tubes on ice, 200 µl of chloroform:isoamyl alcohol (volume ratio 24:1) was added to each tube and the mixture was shaken vigorously for 30 seconds. The solutions were left on ice for 15 min, and then the tubes were centrifuged at 18,000 x *g* for 5 min. The upper clear aqueous phase was transferred to a new tube and an equal volume of isopropanol was added. This mixture was stored at -20°C for at least one hour. Following isopropanol precipitation, samples were centrifuged at 18,000 x *g* for 10 min. The supernatant was discarded, and 1 ml of 75% (v/v) ethanol was added to wash the pellet. Once again, tubes were

centrifuged at 18,000 x *g* for 5 min, and the ethanol solution was removed. The pellet was air dried and re-suspended in 80 µl of nuclease-free water.

2.3.2 DNase treatment of RNA

To remove genomic DNA from RNA preparations, samples were treated with TURBO DNase (Ambion, Austin, TX, USA) using the turbo DNA-free kit (Ambion) according to the manufacturer's instructions. RNA (200 µg per 50 µL reaction), 1 µL Turbo DNase enzyme and 5 µL Turbo DNase buffer (components not disclosed) were combined, mixed by pipetting, and incubated at 37°C for 30 minutes. The enzyme was inactivated using the DNase Inactivation Reagent provided in the kit. The final RNA concentration was assessed by spectrophotometry using a NanoDrop device (Thermo Scientific, Cambridge, UK) (Section 2.3.11).

2.3.3 Reverse transcription

The GoScript Reverse Transcriptase System (Promega) was used to synthesise cDNA from total plant RNA. The manufacturer's protocol was followed; firstly mixing 1 µL random primers (0.5 µg) (Promega), total DNase-treated RNA (up to 5 µg) and distilled water in a PCR tube, to a volume of 10µL. The mixture was heated to 70°C for 5 minutes and placed on ice for 5 minutes. The contents of the tube were collected by brief centrifugation (Tomy PMC-860 Capsulefuge) before adding 4 µL GoScript 5x Reaction Buffer, 3 µl MgCl2, 1 µl dNTPs (Bioline, London, UK) (final concentration 0.5 mM), 1 µl RNaseOUT (Invitrogen) and 1 µl GoScript Reverse Transcriptase. The reagents were mixed by pipetting, collected by brief centrifugation and the reaction was incubated at 25°C for 5 minutes, 42°C for 1 hour and inactivated by heating to 70°C for 15 minutes.

2.3.4 Rapid extraction of plant genomic DNA

Genomic DNA was extracted from flowers of Arabidopsis and *N. benthamiana* plants. From each plant, two to five flowers were collected in a sterile 1.5 ml microfuge tube. Flower material was ground using a micropestle for 15 seconds without buffer. Then, 400 µl of extraction buffer [200 mM Tris HCL pH 7.0, 250 mM NaCl, 25 mM EDTA, and 0.5% (v/v) SDS] was added to the ground tissue. The microfuge tube containing each sample was vortexed and centrifuged at 13,000 x *g* for two minutes. The supernatant ~ 300 µl was transferred to a new 1.5 microfuge tube containing 300 µl of isopropanol. The samples were left at room temperature for 5 minutes, before being centrifuged at 13,000 x *g* for five minutes to pellet the DNA. The supernatant was discarded and the pellet in the tube was left to air-dry for 15 minutes. The pellet was resuspended with 80 µl sterile water. The DNA was quantified using a Nanodrop ND 1000 spectrophotometer and the DNA was diluted where necessary to 20 ng/µl for use in the PCR analyses (Section 2.3.11).

2.3.5 Primer design

For PCR procedures the T_m of the primers was designed to be above 55°C. The difference of T_m between a pair of primers was no more than 5°C apart. The GC concentration of oligonucleotides never exceeded 55%. The primers were designed using the SnapGene® Software. For a complete list of primers used in this project see Appendix 1.

2.3.6 Phusion DNA polymerase

Phusion DNA polymerase is a high-fidelity DNA polymerase that has a decreased error rate during copying. A 50 µL reaction contained 5x HF Phusion buffer (containing a final volume of 1.5 mM $MgCl_2$, 2 µM dNTPs (Promega), 0.5 µM forward

and reverse primers, 1 µL cDNA template (20-50 ng), 0.5 µL Phusion DNA polymerase (0.02 U/µL).

Phusion DNA polymerase was used to amplify regions of virus genomes used for cloning and synthesis of hairpin RNAi constructs (Section 2.5). The PCR cycling conditions were as follows: denaturation at 98°C for 30 seconds, followed by 30 cycles of 5 seconds at 98°C, 20 seconds of 58-62°C (annealing temperature varying according to primer sequences), and 72°C for 1 minute per amplicon length in kb. The program finished with an extension period of 7 minutes at 72°C.

2.3.7 Polymerase chain reaction

PCR was conducted using a pre-mixed reaction mix “Biomix Red” (Bioline). Appropriate primers were designed to carry out colony PCRs, PCR screens to identify the presence of the T-DNA insertion in DNA extracted from transformed plants (see Section 2.3.4). The total reaction volume was 20 µl per sample (10 µl Biomix Red, 0.5 µl (10mM) forward primer, 0.5 µl (10 mM) reverse primer, 8 µl sterilized water and 1 µl DNA template [20 µg/ml]). PCR cycling conditions were initial denaturation at 94°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, an annealing step at 60°C, an extension step at 72°C for 1 minute and a final extension of 72°C for 3 minutes on thermal cycler (Veriti, ABI-Applied Biosystems, USA).

2.3.8 Gel electrophoresis of DNA

The PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels in TAE (Tris-acetate EDTA) buffer [0.04 M Tris, 1 mM EDTA pH 8.0, 0.1142% (v/v) glacial acetic acid] containing 5 mg/ml ethidium bromide. The gels were submerged in TAE buffer and electrophoresis was performed at 80 V for 45 minutes. DNA fragments were visualised under UV illumination on an AlphaImager gel documentation system (AlphaImager, Santa Clara, CA, USA).

2.3.9 PCR product purification

PCR product purification was carried out to remove primers and other PCR components using the MiniElute® PCR purification kit (Qiagen, Hilden, UK) according to the manufacturer's instructions. DNA was eluted in 20 µl of autoclaved distilled water. The concentration was assessed by spectrophotometry using a NanoDrop device (Section 2.3.11).

2.3.10 Extraction of DNA from agarose gel fragments

The DNA band of interest was excised from 1% (w/v) agarose gels using a disposable scalpel and DNA was extracted using the QIAquick® Gel Extraction kit and the provided protocol. DNA was eluted in 50 µl of autoclaved distilled water. The concentration was assessed by spectrophotometry using a NanoDrop device (Section 2.3.11).

2.3.11 Analysis of DNA and RNA concentration and purity

Samples of 1 µL from RNA and DNA preparations were analysed using a NanoDrop 1000 against an appropriate blank (Milli-Q water). The concentration of DNA and RNA in ng/µL was estimated by applying Beer's Law to the absorbance value at 260 nm using an extraction coefficient of either 50 or 40, respectively. The ratio of absorbance at 260 nm to absorbance at 280 nm should be between 1.8 and 2.0 for high purity RNA preparations. A ratio lower than 1.8 indicates either that the sample is contaminated with protein and/or phenol (Glasel, 1995).

2.3.12 Sanger Sequencing

To check the identity of a PCR product or the insert of a sequence into a vector, amplified fragments or plasmid minipreps were sequenced (100ng/µL of plasmid DNA and 1 ng/µL per 100 bp of PCR product). The purified products and 10 µL of the appropriate primer (concentration 10 pmol/ µL) were sent to the sequencing provider (Source Bioscience UK Ltd, Cambridge, UK) for automated Sanger sequencing

(Sanger et al., 1977; Smith et al., 1986). The returned sequencing data was checked using Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990) to confirm that the amplified DNA fragment was as expected.

2.3.13 Phenol-chloroform extraction, precipitation and re-suspension of DNA and RNA

Remaining enzymes and other contaminants were inactivated and/or removed by phenol-chloroform extraction. An equal volume 1:1 phenol:chloroform was added to the DNA or RNA solution, mixed by vortexing and centrifuged at 12,000 x *g* for 10 minutes to resolve phases. The top phase was transferred to a fresh 1.5 mL microcentrifuge tube, which was then precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% (v/v) ethanol. The solution was mixed by inverting the microcentrifuge tube and placing it in a -20°C freezer for at least two hours, precipitating the DNA/RNA. Samples were centrifuged for 10 minutes at 13,000 x *g* and pellets were dried in a vacuum desiccator for 10 minutes. The pellet was redisolved in 50 µL autoclaved distilled water.

2.4 Gateway cloning technique to generate RNAi hairpin constructs

Gateway™ technology is a cloning technique that allows DNA sequences to be inserted into multiple vector systems (Invitrogen, Gaithersburg, MD, USA). This cloning technique is based upon the attachment or "*att*" sequences, found originally in the bacteriophage λ, which are used within Gateway cloning to enable the desired DNA insert to recombine from an entry vector into any destination vector containing the required *att* sites (Landy, 1989). Gateway cloning consists of two cloning steps, the BP and the LR reactions. In the first step, the *att*B site of the insert and the *att*P site of the entry vector recombine in the BP reaction to produce the entry clone with *att*L sites. The presence of the *att*L sites is required for the LR reaction. Therefore,

once the entry clone is produced, Gateway cloning enables the quick recombination of the desired insert into many different destination vectors to be used for protein expression and functional analysis. In this project, Gateway cloning was used to produce hairpin RNAi (hpRNAi) constructs to transform and generate virus-resistant plants using the binary destination vector, pK7GWIWG2 (II) 0 (Karimi et al., 2002). A map of the vector is shown in Appendix 2.

2.4.1.1 Gateway BP reaction

To enable the recombination of the DNA insert into the entry vector, *att*B sites were added to each end of the sequence of interest by two consecutive PCRs. The first PCR adds the half *att*B site and the second adds the remaining half site. Details of primer sequences can be seen in Appendix 1. The entry vector pDONR 221 (Invitrogen) was used to generate entry clones for each of the two constructs (vector map shown in Appendix 2). Phusion PCR programmes were used to add half and then full *att* sites to PCR products for Gateway cloning (Section 2.3.6). The RNA sequences corresponding to the CMV *2b* sequence and the TuMV *P3N-PIPO* transcriptional slippage site were amplified in the first PCR reaction to add half *att* sites and then another round of PCR was performed to add the full *att* sites. Figures 2.1 and 2.2 illustrate how the specific viral sequence regions were amplified to generate HP-RNAi constructs. For HP-RNAi constructs generated, the 2b and P3N-PIPO genomic regions were amplified by PCR from infectious clones RNA2 (pFny209) and p35STuMVGPF, as described in Sections 2.2.7.1 and 2.2.7.2.

The PCR product of each reaction was purified by gel extraction (Section 2.3.10). The BP reaction was done following the manufacturer's protocol (Life Technologies Corporation, 2012). The following components were added to a 1.5 mL microcentrifuge tube at room temperature and mixed by pipetting: 1-7 µL *att*B-PCR product (15-150ng), 1 µL pDONR 221 (200 ng entry vector), 2 µL BP clonase

enzyme mix, and Milli-Q water to a final volume of 10 µL. The BP reaction was incubated for 12 hours at 25°C and 1 µL of proteinase K solution was added to terminate the BP reaction. The reaction was mixed by vortexing and incubated at 37°C for 10 minutes. *E. coli* DH5α competent cells (50 µL) were transformed with 2 µL BP product by heat shock transformation following the procedure described in Section 2.5.1.1. To confirm a successful insertion into the pDONR 221 vector, a colony PCR (Section 2.5.3) was performed in putative transformed bacteria using M13 forward and reverse primers (see Appendix 1). Only bacteria showing positive bands for the amplified region were grown for further plasmid isolation and sequencing (see Sections 2.54 and 2.3.12). The plasmids containing the expected sequences were used for the Gateway LR reaction.

2.4.1.2 Gateway LR reaction

The Gateway LR reaction was used to recombine the RNAi sequences into the binary destination vector pK7GWIWG2(II)0 (Karimi et al., 2002) used for transformation of Arabidopsis and *N. benthamiana*. It was recommended by the manufacturer that the entry clone containing the inserted sequence be linearized using a restriction enzyme before the LR reaction. The entry clones containing the RNAi sequences were linearized using EcoRV (New England Biolabs). The digestion of each entry vector was followed by precipitation and resuspension of the linearized entry vector DNA (Section 2.3.13).

The LR reaction mixture contained the following components: 6 µL entry clone (50-150ng), 1 µL destination vector (150ng), 1 µL TE buffer (pH 8, to a total volume of 8 µL), and 2 µL LR Clonase enzyme mix. The reaction was mixed thoroughly by vortexing and left for 8-12 hours at 25°C and the reaction was terminated by adding 1 µL proteinase K solution and incubation at 37°C for 10 minutes. Following the same procedure as described for the BP reaction, 2 µL LR reaction mixture was

transformed into *E. coli* DH5α competent cells by heat shock and transformed cells plated onto Luria Bertani plates containing spectinomycin (100 mg/mL) (Section 2.5.1.1). Colony PCR of transformed bacteria, plasmid isolation, and sequencing followed the same procedures described for the BP reaction. Primers used for colony PCR corresponded to the intron region and RNAi sequence as depicted in Panel B of Figures 2.1 and 2.2. Plasmids containing the desired RNAi insertions were transformed into *A. tumefaciens* for plant transformation (see Sections 2.5.2, 2.5.5 and 2.5.6).

2.4.2 Transient expression of HP-constructs in *N. benthamiana*

Transient expression of HP-constructs was performed to test RNA silencing. *A. tumefaciens* cells carrying HP-PIPO, HP-2b, pK7GWIWG2(II)0 empty vector, GFP-2b and GFP-TuMV were grown as explained before (Section 2.2.8). Fully expanded leaves of four to five-week-old *N. benthamiana* plants were used for co-infiltration. Individual leaves were co-infiltrated with constructs 1 and 2 as described in Table 2.2 at a final OD_{600} of 0.5. Five days post-infiltration, the infiltrated leaves were observed under a UV lamp. The empty binary vector pK7GWIWG2(II)0, a clone for the GFP-2b fusion protein and GFP-TUMV were available in the lab (Du et al., 2014b; Hunter et al., 2016; Olspert et al., 2015).

Table 2.2 Hairpin constructs co-infiltrated in *N. benthamiana*

Construct 1	Construct 2
HP-PIPO	GFP-TuMV
HP-PIPO	empty vector
HP-2b	GFP-2b
HP-2b	empty vector

HP-constructs designed to induce RNA silencing against the potyviral *P3N-PIPO* transcriptional slippage and the CMV *2b* sequence were co-infiltrated with empty-vectors and GFP-TuMV or GFP-2b vector to evaluate whether the HP-RNAi constructs designed induce RNA-silencing.

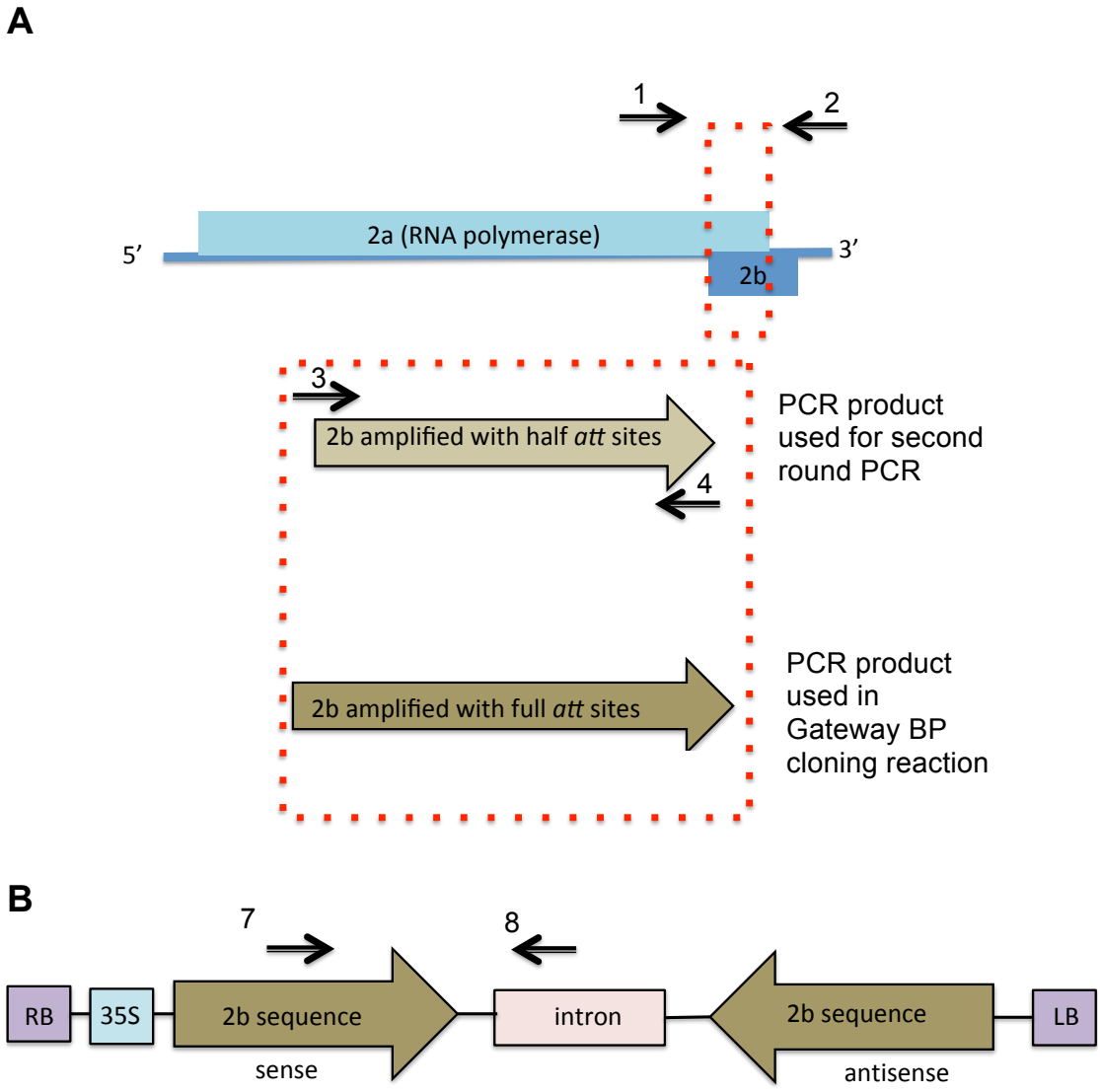


Figure 2.1 Diagram of *2b* genomic region amplified for the HP-2b construct
Panel **A** shows RNA2 of CMV, the region that was amplified by PCR is depicted in the dotted rectangle. The arrows represent the forward and reverse primers used that contained the half *att* sites (primers 1 and 2) and the full *att* sites (primers 3 and 4). Panel **B** shows the sense and antisense orientation of the CMV *2b* sequence and location of intron as inserted into the T-DNA of plasmid pK7GWIWG2(II)0. The diagram also indicates primers 7 and 8 that were used to confirm the successful LR reactions and insertion of the transgene into the plant genome. The sequence of primers is shown in Appendix 1.

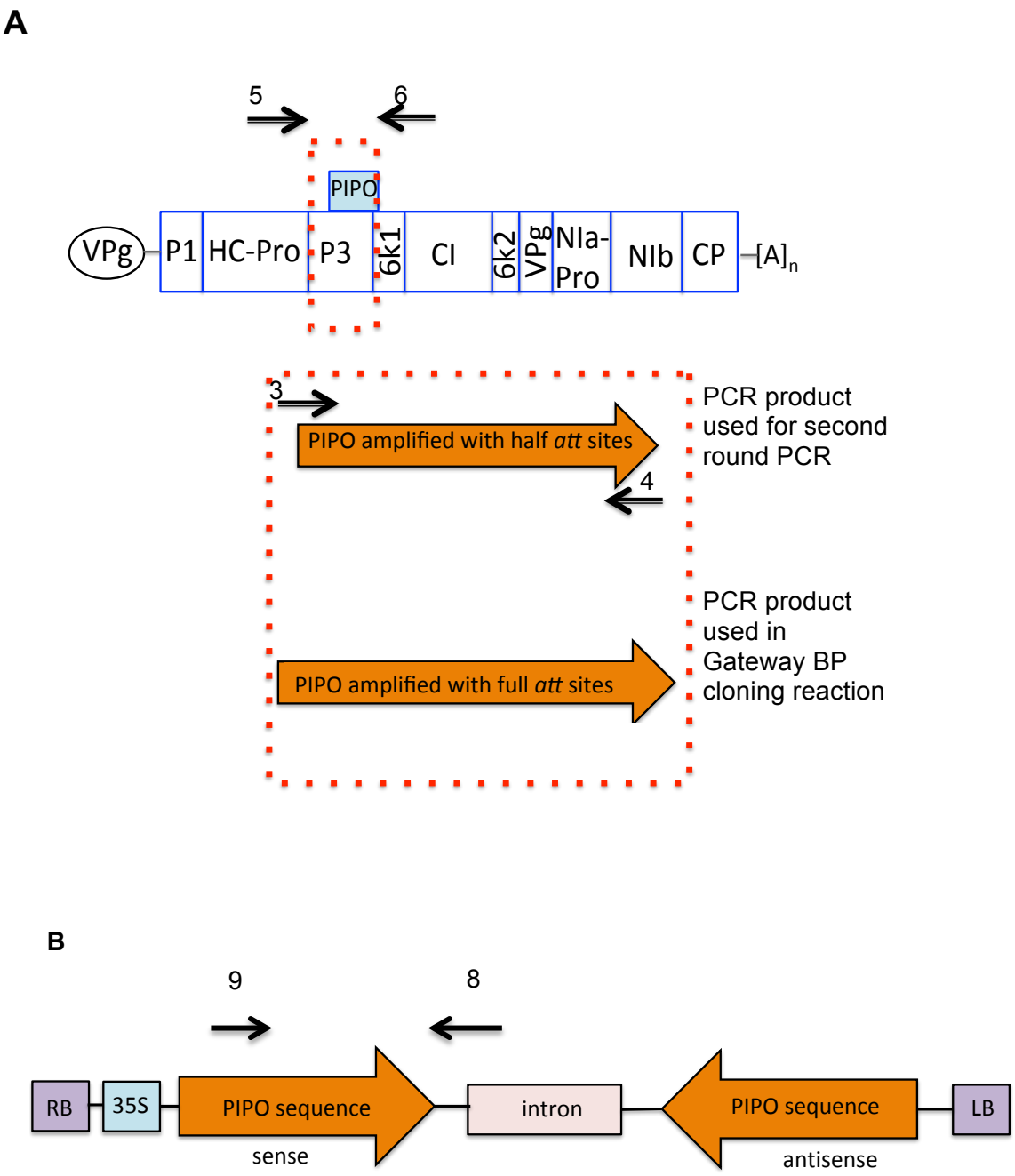


Figure 2.2 Diagram of *P3N-PIPO* transcriptional slippage site amplified for the HP-PIPO construct

Panel **A** shows TuMV genome, and the *P3N-PIPO* transcriptional slippage site that was amplified by PCR is depicted in dotted the rectangle. The arrows represent the forward and reverse primers used in the first round of PCR, which contained the half *att* sites (primers 5 and 6). The PCR product of the first round was then used to add full *att* sites with primers 3 and 4. Panel **B** shows a diagram of sense and antisense orientation of *P3N-PIPO* sequence and location of intron as inserted into the T-DNA of plasmid pK7GWIWG2(II)0. The diagram also shows primers 8 and 9 that were used to confirm successful LR reaction and insertion of the construct into the plant genome. The sequence of primers is shown in Appendix 1.

2.5 Generation of virus-resistant transgenic plants

Arabidopsis plants accessions Col-0 and Ei-2 were used to produce transgenic lines carrying an artificial microRNA or RNAi hairpin constructs designed to provide resistance to CMV and TuMV, respectively (Waterhouse et al., 1998; Smith et al., 2000; Niu et al., 2006). *N. benthamiana* plants were also transformed with the HP-PIPO construct designed to provide resistance to potyviruses.

2.5.1 Bacterial strains

2.5.1.1 Competent *Escherichia coli* cells and heat shock transformation

E. coli DH5α chemically competent cells (Invitrogen) were used for the cloning work and were transformed using the heat shock transformation protocol (Mandel and Higa, 1970). Chemically competent *E.coli* cells in 50 µl aliquots in 1.5 ml microcentrifuge tubes, taken from the -80°C freezer, were allowed to thaw on ice before 1 µl of plasmid DNA was added [100 ng/µl]. The cells were kept for 20 minutes on ice before being placed in a water bath at 42°C for 50 seconds. The *E.coli* cells were returned to ice for 2 minutes before 500 µl SOC medium [SOC: 2% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose] was added. The transformed cells were placed in a 37°C shaking incubator for 1 hour before plating out onto Luria Bertani agar plates containing specific antibiotic for transformants selection. Plates were incubated at 37°C for 24 hours.

2.5.2 *Agrobacterium tumefaciens* transformation

To prepare electrocompetent cells, *A. tumefaciens* LBA4404 cells were taken from homemade stocks and were grown on Luria Bertani agar with rifampicin (50 µg/ml) for 48 hours. A single colony was inoculated in a 100 ml of Luria Bertani with rifampicin (50 µg/ml) and grown at 28°C in a shaking incubator (200 rpm) until the culture reached an OD_{600} of 0.5-1.0. The culture was chilled on ice, transferred to two

50 ml tubes and centrifuged for 5 minutes at 3,000 x *g* at 4°C. The supernatant was discarded and the cells resuspended in 10 ml of sterile 10% (v/v) glycerol under sterile conditions. The cells were spun down and resuspended in 10% (v/v) glycerol and centrifuged three more times. After the last centrifugation, the cells were resuspended in 1 ml 10% (v/v) glycerol. The bacteria suspension was then dispensed into 50 µl aliquots and immediately vitrified in liquid nitrogen. Aliquots were stored at -80°C until needed.

Electroporation was used to transform *A. tumefaciens* strain LBA4404 with the RNAi constructs to be used for plant transformation (An et al., 1989). Prepared aliquots of LBA4404 cells were taken from the -80°C freezer and thawed on ice. Then, 1 µl plasmid DNA [100 ng/µl] was added to 50 µl of LBA4404 electrocompetent cells. The cells were electroporated in a Gene Pulser XCell^{TM} (BIO-RAD) in a 2 mm GenePulser cuvette (BIO-RAD), at a capacitance of 25 µF, 2.4 V and 200 Ω. The cells were incubated in 1 ml of SOC media and incubated at 28°C for 2-4 hours with shaking at 250 rpm. The cells were plated onto Luria Bertani agar plates containing spectinomycin (100 mg/mL) and rifampicin (50 µg/ml), and incubated at 28°C for 48 hours. Transformed colonies appeared on selection plates after 48 hours of incubation. The destination vector pK7GWIWG2(II)0 (Karimi et al., 2002), containing the RNAi hairpin inserts, was used for Arabidopsis and *N. benthamiana* transformation (Sections 2.5.5 and 2.5.6). Vector map is shown in Appendix 2.

2.5.3 Identification of transformed bacterial cells

Putative transformants were selected as white colonies grown on antibiotic selection media. Individual colonies were pricked into 20 µl of distilled water using a sterile toothpick. The cells were incubated at 95°C for 5 minutes. Then, 1 µl of each selected colony was checked for the presence of the insert by PCR (see Section 2.3.7). The colonies that were positive for the desired insert were grown for plasmid

purification (see Section 2.5.4). Plasmid purification was performed and the plasmids were sent for sequencing to confirm the sequence was as expected (Sections 2.3.12 and 2.5.4).

2.5.4 Plasmid purification

Plasmid DNA was extracted from putative transformant *E. coli* cells grown in 5 ml Luria Bertani for 16 hours using a QIAprep® Spin Miniprep Kit following the manufacturer's protocol. Plasmids were eluted in 50 µl of autoclaved Milli-Q water. The concentration was assessed by spectrophotometry using a NanoDrop device (Section 2.3.11).

2.5.5 Floral dip transformation

The procedure is based on that of Clough and Bent (1998). *A. tumefaciens* cells carrying the amiR-SD-3, HP-PIPO and HP-2b constructs respectively were streaked out onto solid Luria Bertani medium with 50 µg/ml kanamycin and 25 µg/ml gentamycin for 48 hours. One colony was inoculated into 2 mL of Luria Bertani medium containing 50 µg/ml kanamycin and 25 µg/ml gentamycin in a 15 ml clear tube and incubated for 24 hours at 28°C in a rotary shaker at 230 rpm. The starter culture was poured into 50 ml Luria Bertani with 50 µg/ml kanamycin and 25 µg/ml gentamycin in a 500 ml conical flask and incubated for 24 hours at 28°C in a rotary shaker at 230 rpm. These cells were pelleted by centrifugation at 6,500 x *g* for 20 minutes at 4°C. The pelleted cells were resuspended in freshly made dipping solution [5% (w/v) sucrose, 0.05% (v/v) of Silwett L77]. The plants were immersed within the dipping solution for 3-5 minutes and kept in dark conditions for 24 hours. Dipped plants were placed under controlled growth room conditions at 22°C with 16-hour photoperiod and allowed to flower to produce seeds. To trigger the plants to produce multiple secondary bolts, the inflorescences were cut off when the plants had formed primary bolts. Approximately two weeks later, the secondary flowering stems of 10-15 cm in length were used for dipping. T1 or T2 generation harvested seeds (50 mg

~2000 seeds) were sown on Murashige and Skoog agar (MSA) medium containing antibiotics as follows: kanamycin (50 mg/ml) for the HP-2b and the HP-PIPO constructs or hygromycin B (20 mg/ml) for the ami-SD-3 construct to select for transformants. The number of resistant plants was scored from each independent event. Antibiotic-resistant seedlings were transferred to growth room conditions at 22°C with 16-hour photoperiod and let to self-fertilize to obtain T2 generation seeds. DNA extraction from putative transformant plants followed by PCR was carried out to determine the presence of the transgene (see Section 2.3.7).

2.5.6 Transformation of *Nicotiana benthamiana*

A. tumefaciens cells carrying the HP-PIPO construct were streaked onto solid Luria Bertani medium containing 50 µg/ml kanamycin and 50 µg/ml rifampicin and incubated for 48 hours. One colony was inoculated into 2 mL of Luria Bertani medium containing 50 µg/ml kanamycin and 50 µg/ml rifampicin in a 15 ml clear tube and incubated for 24 hours at 28°C in a rotary shaker at 230 rpm. The starter culture was poured into 50 ml Luria Bertani with kanamycin (50 µg/ml) in a 500 ml conical flask and incubated for 24 hours at 28°C in a rotary shaker at 230 rpm. These cells were pelleted by centrifugation at 6,500 x *g* for 20 minutes at 4°C and resuspended in Murashige and Skoog medium supplemented with 3% (w/v) sucrose and 150 µM acetosyringone.

All the procedures for plant transformation were carried out under sterile conditions. Healthy *N. benthamiana* leaves of 4-week-old plants were sterilized for 15 minutes in 10% (v/v) hydrochloric acid and rinsed three times with autoclaved distilled water under sterile conditions. The leaves were maintained in autoclaved distilled water while each leaf was cut into 5 mm squares and placed in the resuspended *A. tumefaciens* described above. The immersed cut leaves were then placed on sterile filter paper to remove excess solution and placed on MSA plates supplemented with

0.1 mg/ml naphtalene acetic acid (NAA) and 1.5 mg/ml 6-benzyl amino purine (BAP) for 48 hours under dark conditions. After the incubation period, the leaf explants were passaged to fresh MSA media supplemented with 0.1 mg/ml NAA, 1.5 mg/ml BAP, 100 µg/ml kanamycin and 250 µg/ml gentamicin. Transformed cells regenerated and formed callus tissue. Callus tissue was moved onto fresh MSA plates every two weeks. After several passages, new shoots were produced by the calli. Individual shoots of about 4 cm in length were transferred to rooting media [MSA supplemented with 3% (w/v) sucrose]. Plants that showed root formations were planted in pots containing Levington M3 compost and grown as described before in Section 2.2.2. To detect the transgene, DNA was extracted and PCR was carried out with appropriate primers (see Sections 2.3.4 and 2.3.7).

2.5.7 Viral inoculation of transgenic lines

2.5.7.1 Mechanical inoculation

To assess the level of virus resistance the plants were challenged with purified virions 24 days after germination, as described in Section 2.2.9. Symptoms of the inoculated plants were recorded 7, 14 and 21 days after inoculation. Three weeks after inoculation the plants that showed mild and no viral symptoms were analysed by ELISA (see Section 2.2.12).

2.5.7.2 Aphid-inoculation for virus transmission

To assess the level of virus resistance by aphid-inoculation, the plants were arranged in 3x3 arrays and a virus-infected plant (referred to as “source plant” in this project) was placed in the centre (Figure 2.3). The day before aphid-inoculation, thirty (seven-day-old) aphids were collected in a 5 cm plastic Petri dish and starved for 10-12 hours by keeping them at 4°C. Fine-tip paintbrushes were used to transfer the adult aphids from aphid stock plants to the Petri dishes and experimental plants.

On the day of the experiment, the plastic Petri dishes containing the aphids were kept at room temperature for 1 hour to acclimatise. Then, the aphids were placed on the source plant and each array was covered with a micro-perforated plastic bag to contain the aphids. After 24 hours a systemic neonicotinoid insecticide ("Intercept 70 W", Everris Ltd, UK; imidacloprid; 0.5 g/l) was applied to the soil around the plants to kill the aphids. Three weeks after inoculation the plants that showed mild or no apparent viral symptoms were analysed by ELISA (see Section 2.2.12).

2.5.8 Assessment of virus resistance

The methods described in Section 2.5.7 were used to inoculate virus-resistant plants via mechanical or aphid-inoculation. For each independent line a total of 15 to 20 plants were inoculated mechanically with virions at a concentration of 20 µg/ml for CMV or 5 µg/ml for TuMV. The degree of resistance (% of plants displaying resistance) for each independent line was evaluated with the formula shown below.

$$\%\ of\ resistant\ plants\ = \frac{Total\ number\ of\ non\ infected\ plants}{Total\ number\ of\ inoculated\ plants}\ x\ 100$$

Only the lines that showed virus resistance of 70% or greater were further assessed for virus resistance by aphid-inoculation. For aphid-inoculation a total of 3 to 5 arrays (3x3 plants) were assessed (Section 2.5.7) and the proportion of resistant plants was also calculated with the formula described above. TuMV-inoculated plants were also observed under a UV lamp because GFP-TuMV was used for virus inoculation. Only plant lines that displayed high resistance (70% or greater) in both mechanical and aphid-inoculation were used to evaluate aphid-settling responses (Section 2.5.9).

2.5.9 Characterisation of virus-resistant plants for aphid experiments

Selected virus-resistant plants were grown along with wild-type plants for comparison until flowering time. Any visual differences with the wild-type plants were noted to discard lines. Healthy plants of lines that showed resistance by mechanical and aphid-inoculation (Section 2.5.7.1 and 2.5.7.2) were assessed with aphid assays such as mean relative growth rate (MRGR) and settling choice test described in Section 2.6 to determine if the transformation process had altered their susceptibility or attractiveness to aphids.

2.6 Aphid performance assays

2.6.1 Mean Relative Growth Rate

One-day-old-aphid nymphs were individually weighed on a microbalance (MX5, Mettler Toledo, Columbus, OH, USA) before being placed on test plants (Arabidopsis Col-0, Ei-2, Ler, Cvi). Nymphs were contained on experimental plants using micro-perforated plastic bags secured at the base of each pot with an elastic band (1 nymph per plant). The final weight of each aphid was measured five days post-infestation. Then, the mean relative growth rate (MRGR) was calculated using the formula:

$$MRGR = \frac{\log \quad W_{final} - \log \quad W_{initial}}{t}$$

where t= time is the number of days between initial and final measurements of aphid fresh weight (W) (Leather and Dixon, 1984). At least 20 replicates per treatment group were used and experiments performed at least three times unless otherwise stated. Fine-tip paintbrushes were used to transfer the nymphs from Chinese cabbage plants to Arabidopsis plants.

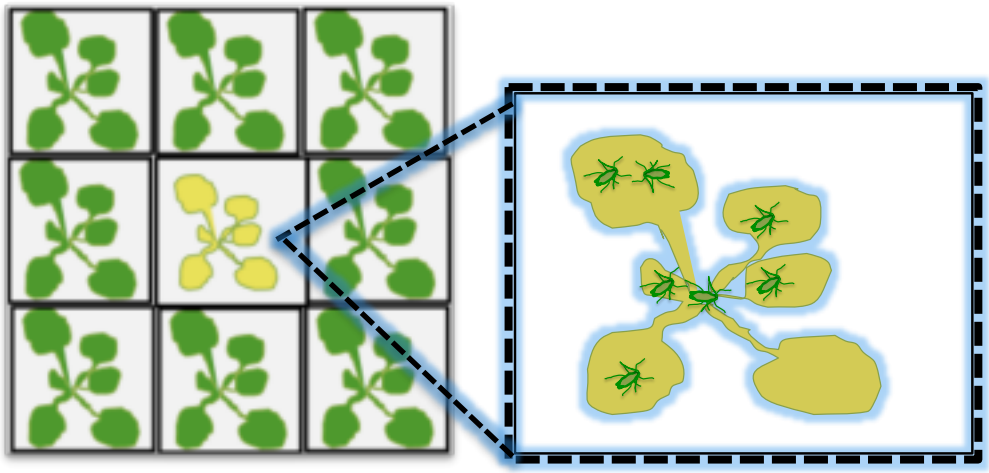


Figure 2.3 Diagram of assay set-up for virus transmission by aphids

Thirty (seven-day-old) aphids were placed on top of the virus-infected source plant (yellow plant) to acquire virus particles. After 24 hours, when the aphids had freely moved to healthy/susceptible plants (green plants) insecticide was applied to stop further transmission.

2.6.2 Aphid colony growth

Adult aphids were left for 24 hours to reproduce on aphid free Chinese cabbage plants. The nymphs produced were then transferred to experimental plants using a fine-tip paintbrush. For aphid colony growth assays, one-day-old nymphs were placed on test plants (one insect per plant). The number of nymphs produced was recorded at 10 days post-placement on the Arabidopsis plants.

2.6.3 Migration of viruliferous and non virulifeorus aphids

Arabidopsis plants were arranged in rows of 16 plants (Figure 2.4). The initial plant (position 0, Figure 2.4) was inoculated with CMV (100 µg/ml) or mock-inoculated with distilled water 24 days after germination (Section 2.2.9). Seven days after inoculation, six 7-day-old aphids were placed on top of the source plant (CMV-infected or mock-inoculated). Each row was enclosed into a perforated plastic bag (120x15 cm) and placed into a cage. After 24 hours, the number of aphids was counted on each plant along the row to assess the extent of movement of the aphids away from the source plant (plant in position 0) (Figure 2.5). After the assessment, the plants were treated with insecticide to kill the aphids and to prevent further virus transmission. The plants were left to grow for another 2-3 weeks to allow the appearance of symptoms. Plants were analysed using ELISA to confirm the presence of the virus (Section 2.2.12). Each experiment consisted of 14 rows with a mock-inoculated source plant and 14 rows with a virus-inoculated source plant, unless otherwise stated.

2.6.4 Starvation and number of probes assays

Starvation assays were performed to assess the effect of starvation on the number of probes that an aphid made on Arabidopsis plants over 5 minutes. Adults (seven-day-old) *M. persicae* were removed from Chinese cabbage and starved in a Petri dish for 10-12 hours at 4°C. On the day of the experiment, Petri dishes containing aphids were kept for 1 hour at room temperature for acclimatisation.

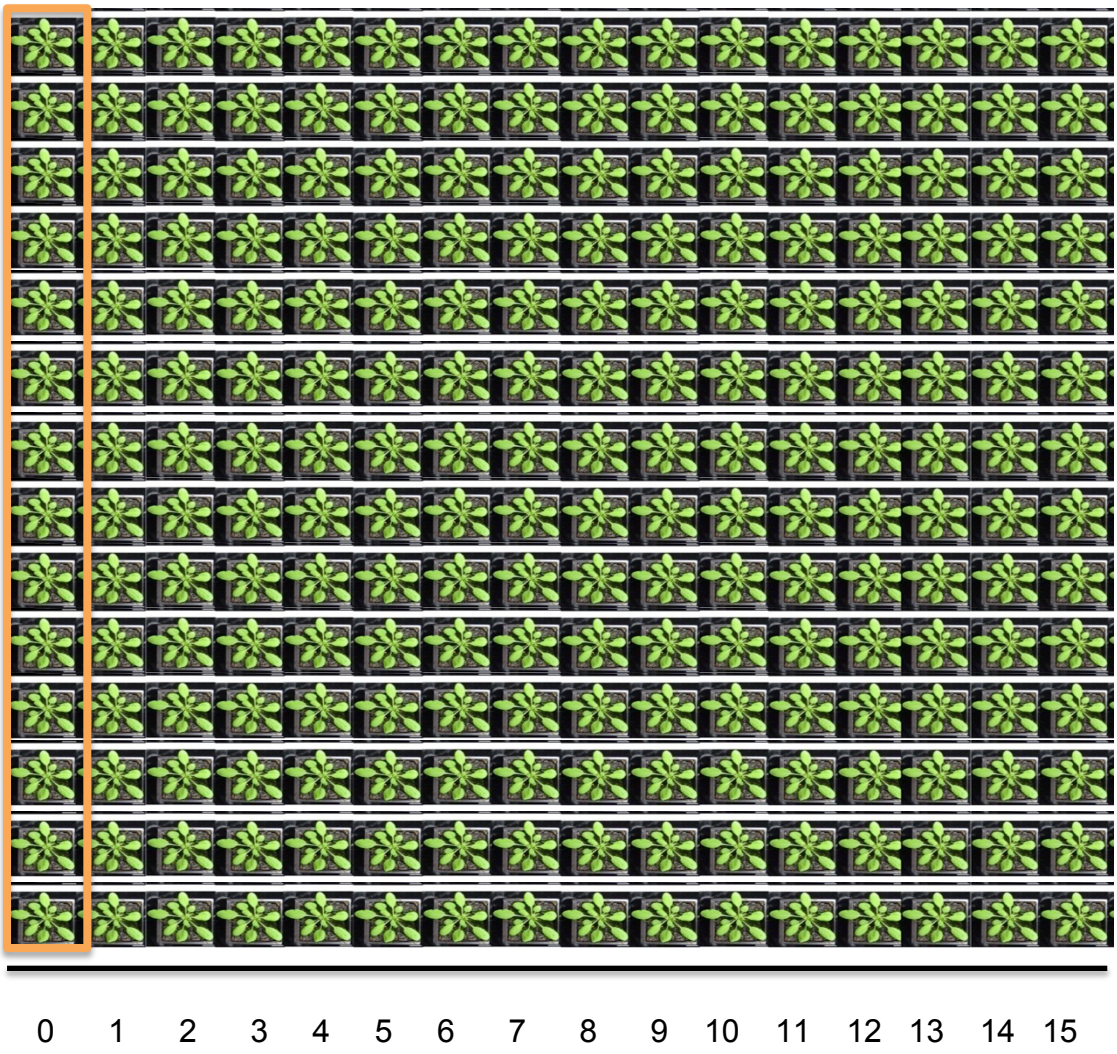


Figure 2.4 Set-up for line experiments to monitor aphid migration
A total of 16 Arabidopsis plants were arranged in a row. The initial plant (plant position 0 or source plant) was either mock-inoculated or inoculated with CMV. An experiment consisted of 14 rows with a mock source plant and 14 rows with Fny-CMV inoculated source plant. Seven days after inoculation, 6 seven-day-old aphids were placed on each initial plant per

A single pre-starved adult aphid was placed on a CMV-infected plant and observed through a magnifying lamp (1.75X) for five minutes to assess the number of probes each aphid made. A probe was recorded each time an aphid moved its antennae backwards over the abdomen, a behaviour, which has been reported as indicating the start of plant probing (Caillaud et al., 1995). Each probe was defined as finished when antenna and body movement were resumed. This behaviour has been previously reported for *M. persicae* stylet penetration (Powell et al., 1993). Powell and colleagues (1993) defined stylet penetration for *M. persicae* as the backward movement of the antennae following immobilisation of the head and body, stylet penetration finishes when head and body movement are resumed (Powell et al., 1993).

2.6.5 Olfactometry assays

Two-way olfactometry was conducted to test whether aphids showed a preference for plants based on olfactory cues (Du et al., 1996). A glass Y-tube olfactometer with 8 cm long arms (Internal diameter 1.5 cm) and main arm of 9cm was used for olfactometry assays. Charcoal-filtered air was pumped through Teflon tubing and divided by a T-junction to obtain two airflows that passed through two separate flow meters, which were regulated to function at 400 ml/min flow rate. The air passed into two separate collection vessels (8.5 cm diameter and 7 cm height) into which the pots containing plants to be tested were placed. Each pot contained a metal dish with 19 holes where single Arabidopsis plants were grown and mock-inoculated or virus-inoculated after 3 weeks of growth (Section 2.2.9). The pots containing the plants were kept under the same conditions described above for Arabidopsis plants (Section 2.2.4). After 10 days of infection the pots were used for olfactometry assays. A total of 25 aphids were introduced into the main arm of the Y-tube with the aid of a fine paintbrush and aphids were given 1 hour to make a choice. The time was chosen to match the 1-hour assessment of settling choice tests (see Section 2.6.7).

Only aphids that reached the end of each arm were considered for analysis. A fresh pair of pots containing Arabidopsis plants and clean glassware were used for every sample. A diagram of the olfactometry setup is shown in Figure 2.5. Visual cues from plants were masked by placing the Y-tubes at the same level of pots i.e the pots containing the plants were above the sight level of aphids because the Y-tube was located 15 cm below the pots containing Arabidopsis plants. The experiment was performed in a Faraday cage to provide constant light conditions and avoid other light sources that might affect aphid responses.

2.6.6 Adhesive trap assay to study aphid host location

Aphid host location was examined using a two-way choice test. Two Arabidopsis plants were placed in 15x15 cm square pots. Plants were virus-inoculated or mock-inoculated after three weeks of growth. The plants used for choice assays were at 10 days post-inoculation. Adult aphids (7-day-old) were collected from Chinese cabbage stock plants and placed into a 1.5 ml microfuge tube using a fine paintbrush. The aphids were starved for 10-12 hours at 4°C. The following day, microfuge tubes containing aphids were moved to room temperature for 1 hour for acclimatisation. Aphids (25-30) in a microfuge tube were placed in the centre of the arena, next to the plants sections of adhesive tape (Q-Connect double-sided tape) were placed to trap aphids before the aphids had reached the plant that they were moving towards (Figure 2.6). The square pots were covered with a microperforated plastic bag secured with an elastic band to ensure aphids remained within the arena. The number of aphids caught on the adhesive traps was assessed every 10 minutes throughout one-hour observation. The experiment was performed under both light and dark conditions. For the dark conditions, 15x15 square pots were placed upside down to function as “lids” to provide the dark conditions. Then the “lid” was removed to count aphids and put it back after counting. Ten biological replicates were performed per treatment and the experiment was performed three times.

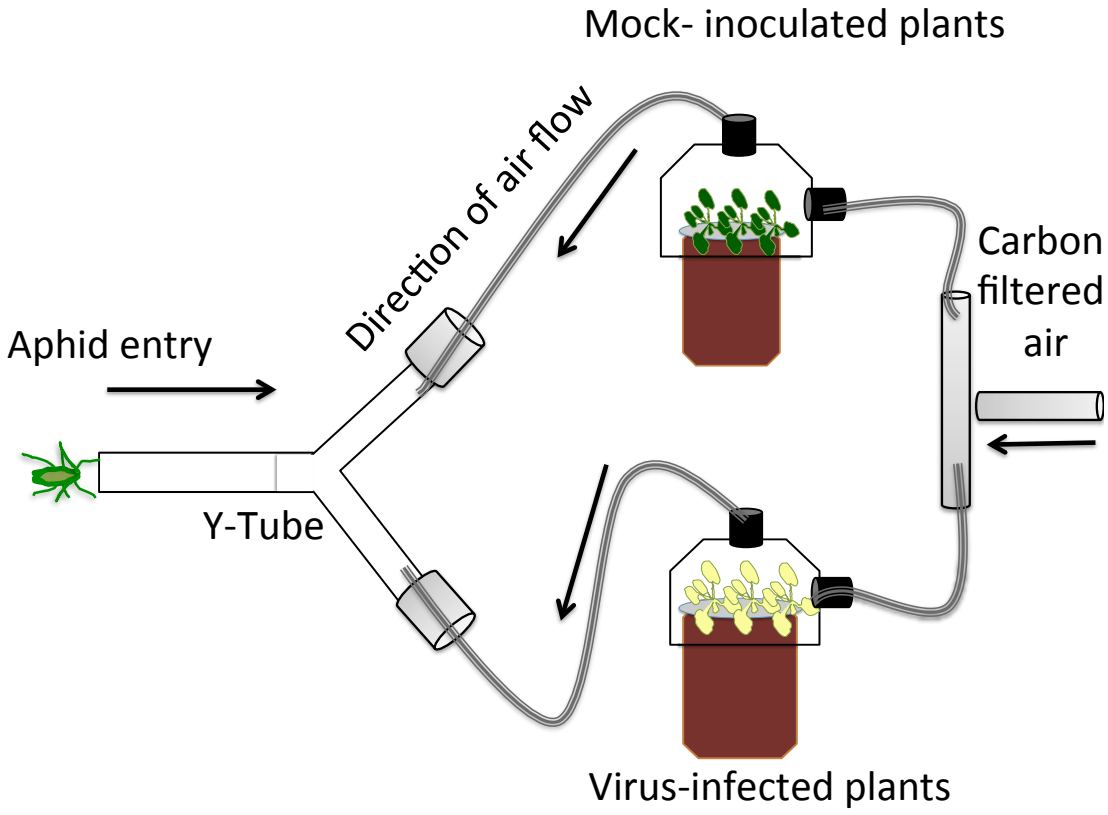


Figure 2.5 Diagram of olfactometry set-up

Pots containing virus-infected plants or mock-inoculated plants were placed inside glass vessels. Charcoal-filtered air was pumped to pass through the two glass containers and then into the two arms of the olfactometer. Seven-day old aphids were released into the main arm of the Y-tube. Aphids were exposed to the emitted odours and their movement monitored.

Figure 2.6 Adhesive trap assays: set-up for aphid host location assays
Two plants were placed in 15x15 cm square pots. A microfuge tube containing 25-30 aphids was placed in the centre of the arena. Aphids were allowed to move freely. The picture shows a representative arena used for choice tests to determine aphid host location. Only aphids found on each adhesive trap were counted and considered for statistical analysis. Scale bar, 1 cm.

2.6.7 Electrical Penetration Graph (EPG) assays to assess aphid probing behaviour

The direct current electrical penetration graph (DC-EPG) method was used to assess aphid-probing behaviour. The method was developed to study plant-aphid interactions at the plant tissue level (Tjallingii, 1978).

EPG was used to monitor aphid-probing activity on virus-infected and mock-inoculated Arabidopsis accessions Col-0 and Ei-2. Adult aphids (7-day-old) were pre-starved for 60 minutes prior to being dorsally tethered to a 4 cm gold wire (20 µm diameter) (EPG systems, Wageningen, the Netherlands) using water-based conductive silver paint. The gold wire was soldered to a 2 cm brass pin connected to an amplifier having a 1 GΩ input resistance and a moderate 50-100 x gain. The "plant" electrode (a 2 mm diameter, 10 cm long copper rod) was inserted into the plant-potting medium. Then plant and aphid were made part of an electrical circuit with the amplifier. The system was kept in a Faraday cage to limit electrical interference. The EPG system was set up with a computer running Probe 3.4 software (EPG Systems) on Microsoft Windows. Once the recording was begun the connected aphids were lowered onto the adaxial side of Arabidopsis rosettes. Aphid feeding behaviour was recorded for 8 hours, starting at 9 am each time to maintain the same light conditions in which the aphids were reared (Section 2.2.6) The recordings were exported from Probe 3.4 software to A2EPG software to visualise and analyse the electrical signals (Adasme-Carreño et al., 2015). The waveforms were scored according to previously described methods and EPG parameters were calculated using Microsoft Excel-based spreadsheets developed for automatic parameter calculation of EPG data (Sarria et al., 2009). A schematic diagram of EPG is shown in Figure 2.8. A summary of the typical waveforms related to aphid feeding behaviour is depicted in Table 2.3.

Table 2.3 Typical EPG waveforms related to aphid feeding behaviour

EPG Waveform	Description
Not probing (np)	Aphid stylet is not inserted into plant tissue
Pathway activities (C)	Stylet pathway phase, including four pooled pathway waveforms/activities, i.e., waveform A, epidermis first stylet contact; and subsequent cyclic activity of waveform B, inter-cellular sheath salivation; waveform C, stylet movements; and waveform pd (potential drop), an intracellular stylet puncture of epidermal and mesophyll cells
Phloem salivation (E1)	Excretion of saliva into the phloem preceding phloem ingestion
Phloem ingestion (E2)	Passive ingestion of phloem sap following salivation into the phloem
Probing difficulties (F)	Derailed stylet mechanics (stylet penetration difficulties)
Xylem ingestion (G)	Active drinking from xylem

Adapted from (Tjallingii and Esch, 1993)

2.6.8 Choice test to study aphid settling responses

The preference of aphids for settling on different host plants was examined using a two-way choice test (Figure 2.7A). The set-up was similar to that described in Section 2.6.6, but adhesive traps were not used and the experiments were only performed under light conditions. Depending on the experiment, plants were used at 3, 9, or 21 days post-inoculation. Ten biological replicates were performed per treatment and the experiment was performed three times.

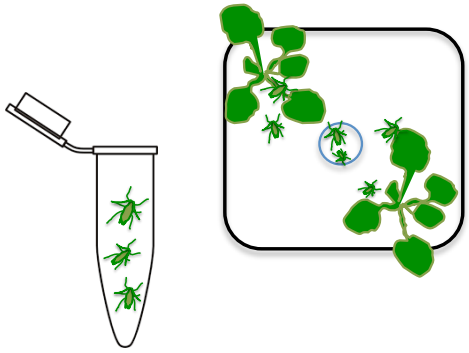
2.6.9 Multi-way choice test to determine aphid settling responses

The preference of aphids for more than two plants accessions or treatments was performed in a multi-way choice test. Eight Arabidopsis plants were arranged in a circle in a 20-cm diameter pot (Figure 2.7B). Plants were virus-inoculated or mock-inoculated after three weeks of growth. After 10 days of inoculation, in the centre of the arena 25-30 aphids (7-day old) were placed in a 5 cm Petri dish. Each pot was enclosed in a plastic bag and placed in a cage. After 1 hour and 24 hours, the number of aphids was counted on each plant. A total of 10 biological replicates were performed per treatment and the experiments were performed three times.

2.7 Microcosm experiments to study plant-virus-vector interactions

Two-dimensional experiments were set up to assess manipulation of aphid behaviour and transmission of two non-persistently transmitted viruses (CMV and TuMV). In this thesis I will refer to this type of experiment as a “microcosm experiment”. A microcosm was composed of an array of 25 plants. A virus-infected plant (source plant) was placed in the centre and was surrounded by two layers of healthy/susceptible plants (Figure 2.9). To randomize the position of transgenic plants or different Arabidopsis accessions the following command in R was used: sample (1:24, number of plants to be randomized, replace =FALSE).

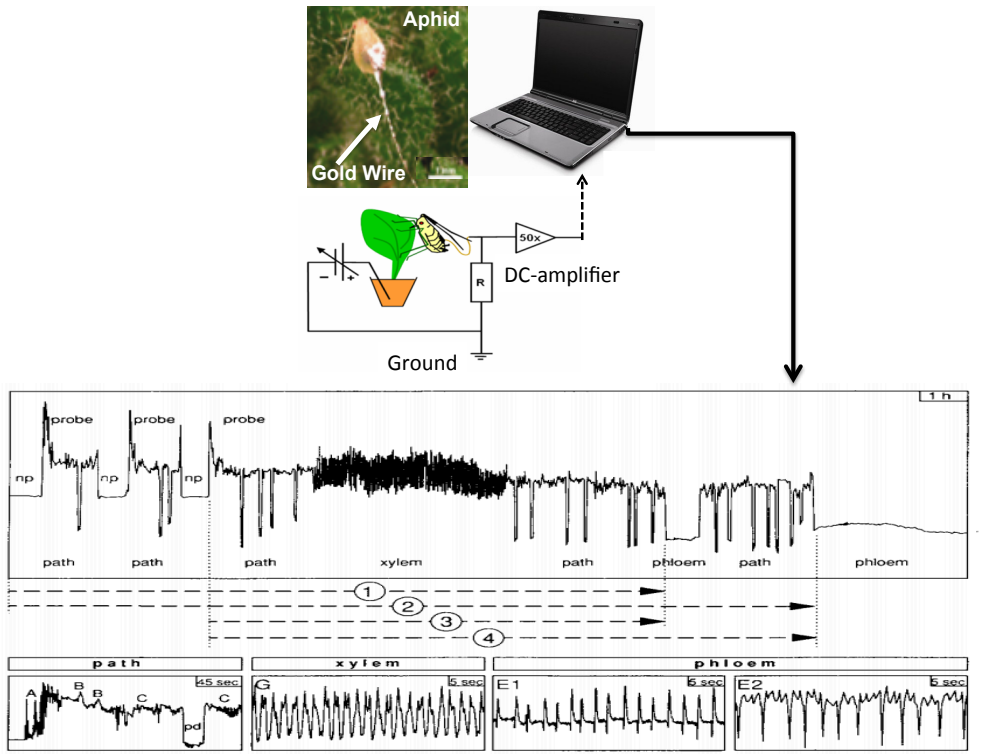
A



B

Figure 2.7 Choice test set-up to assess aphid settling responses

Panel **A** is a diagram for a two-way settling choice test. Two plants were arranged into opposite corners of a 15x15 cm square pot. A microfuge tube containing 25-30 starved aphids (7-day old) was located in the centre of the arena and pushed into the compost so it was levelled with the substrate surface. Once the tube was levelled the lid was open to release aphids to choose a suitable host. Panel **B** shows pictures of representative pots used for multi-way settling choice test. A total of 8 plants (2 plants per treatment) were used per pot and 25-30 aphids were placed in the centre of the arena. The plants were arranged in an alternating manner. Scale bar, 1cm.



Adapted from (Tjallingii and Esch, 1993; Tjallingii, 2006)

Figure 2.8 Schematic diagram of EPG set-up and phloem feeding waveforms

Aphids are attached to a gold wire with silver glue and placed on the adaxial side of an Arabidopsis leaf. Aphid stylet penetration into the plant creates a circuit that is recorded and interpreted as a signal using an EPG software package run in a computer. The voltage fluctuations display distinct patterns in regard to amplitude, frequency and voltage levels, which represent the EPG waveforms. Each waveform is correlated with aphid stylet position in plant tissues (Table 2.3). Typical EPG waveforms are shown which represent different aphid stylet positions within plant tissue.

Aphids were allowed to move freely from the source plant as previously described (Section 2.5.7.2). After 24 hours, the number of aphids found on each plant was recorded. Insecticide was applied to each microcosm and plants were kept for another 2-3 weeks to allow the development of viral symptoms. Plants were analysed using ELISA to confirm infection (Section 2.2.12). The proportion of aphids found on the source plant, first layer and second layer of plants was evaluated with the formulas shown below.

i)

$$\%\ of aphids\ on\ source\ plant\ = \frac{Total\ number\ of aphids\ found\ \ on\ source\ plant}{Total\ number\ of aphids\ found\ per\ array}\ x\ 100$$

ii)

$$\%\ of\ aphids\ on\ first\ layer\ = \frac{Total\ number\ of aphids\ found\ \ on\ first\ layer}{Total\ number\ of\ aphids\ found\ per\ array}\ x\ 100$$

iii)

$$\%\ of\ aphids\ on\ second\ layer\ = \frac{Total\ number\ of aphids\ found\ \ second\ layer}{Total\ number\ of\ aphids\ found\ per\ array}\ x\ 100$$

The proportion of symptomatic plants counted in each microcosm was calculated with the following formula.

$$\%\ of\ symptomatic\ plants\ = \frac{Total\ number\ of\ symptomatic\ plants}{Total\ number\ of\ plants}\ x\ 100$$

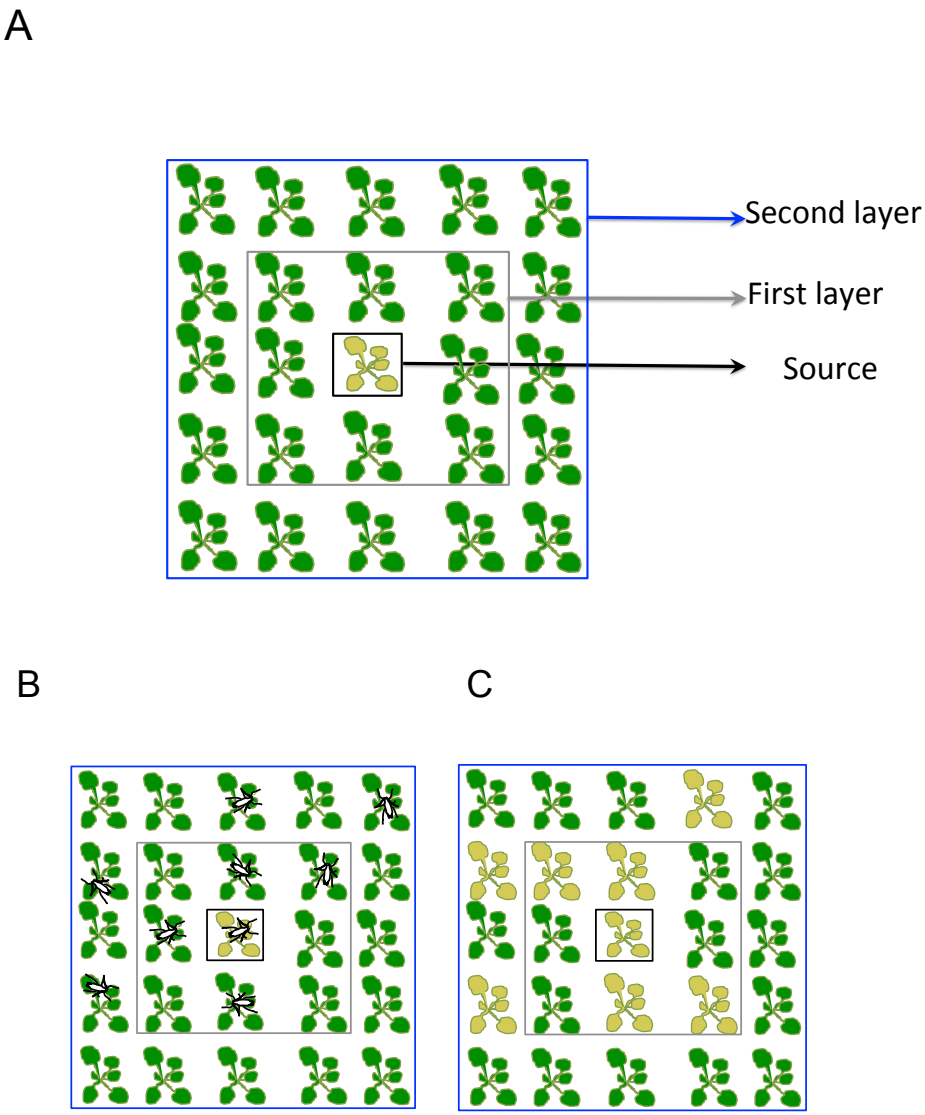


Figure 2.9 Microcosm experiments to study plant-virus-vector interactions

A total of 25 plants were arranged in a 5x5 array (microcosm). **A**. Virus-infected source plants were placed in the centre and surrounded by two layers of healthy plants. **B**. Aphids were placed on the source plants and allowed to move freely for 24 hours before insecticide application. **C**. Virus transmission on healthy plants was assessed after two weeks of aphid-inoculation by observation of symptoms and ELISA.

2.8 Volatile organic compound analysis

2.8.1 Collection of volatile organic compounds by air entrainment of headspace volatiles

The blend of VOCs emitted by Arabidopsis plants (mock or CMV-inoculated) was examined using headspace analysis following a procedure described before (Beale et al., 2006) and shown in Figure 2.10 (equipment was manufactured at Rothamsted Research). Arabidopsis plants grown as described in Section 2.2.4 were pricked out in 9 cm metal dishes containing 19 holes. After two weeks of growth, plants were mock- or CMV-inoculated (Section 2.2.9) and experimental pots were used at 10 dpi. Each VOC collection was performed in pairs (one infected-plant and one mock-inoculated plant). A total of three pairs of pots were used for Col-0 and Ei-2 plants. For VOCs emitted by transgenic plants (Section 3.2.7), the VOC collection was performed in pairs (one transgenic plant and one non-transgenic plant both in Col-0 background) with a total of three pairs of pots for ami-SD-3 1.12 and non-transgenic Col-0 plants. Glassware and metal plates used for volatiles collections were washed in Teepol (0.1% v/v) to remove organic residues and rinsed with distilled water, followed by 100% acetone to remove ionic residues and baked at 180°C for 2 hours. Each pot containing metal plates with Arabidopsis plants was placed in a glass chamber (8.5 cm diameter and 7 cm height) and secured with polytetrafluoroethylene (PTFE) tape and bulldog clips. Charcoal filtered air was pumped in via a tube fixed to an inlet port at the bottom of the glass vessel at a rate of 800 ml/min. Air was removed from the glass chamber at a rate of 700 ml/min through a Porapak Q Filter (50 mg, 50/80 mesh size, Supelco (Sigma-Aldrich)) contained inside a glass gas chromatograph inlet liner between two plugs of glass wool. The volatile collection was carried out for 24 hours and it was always started at 9 am in the morning to the keep same light conditions in which the plants were growing (see Section 2.2.4). The entrained VOCs were eluted from the Porapak Q Filter using 700 µl of diethlyl ether.

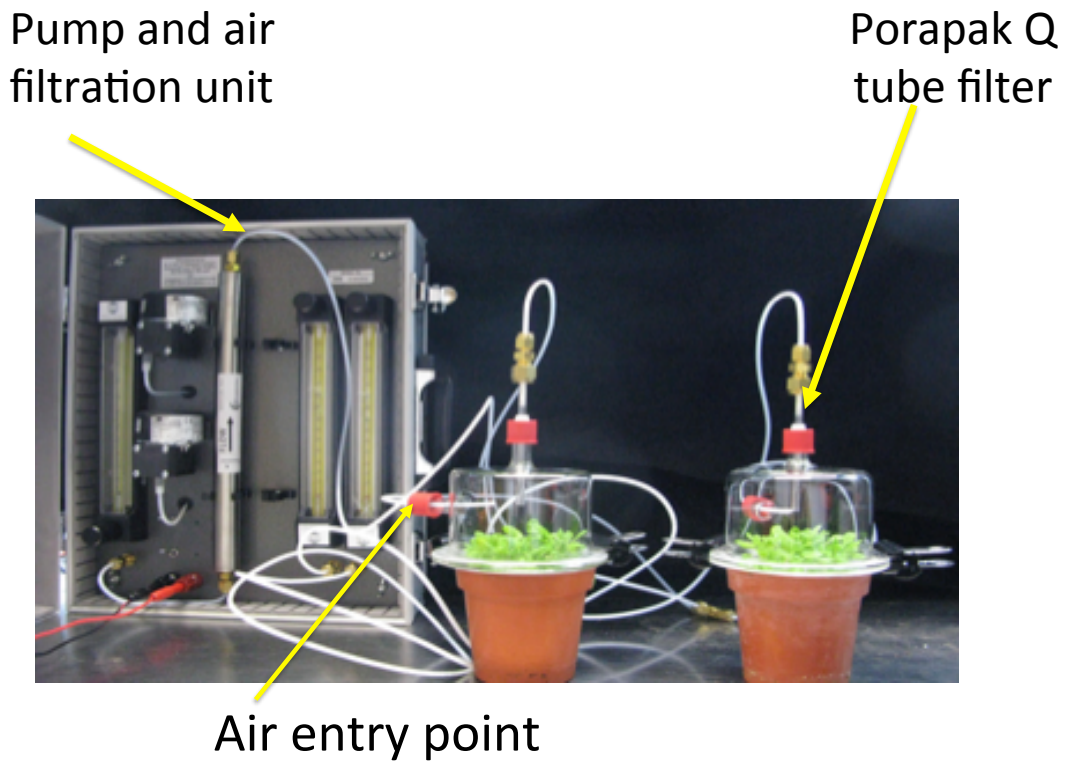


Figure 2.10 Set-up used for headspace entrainment
Two pots containing mock-inoculated and CMV-infected Arabidopsis plants were contained in glass vessels. Headspace volatiles were collected over a period of 24 hours. Charcoal-filtered air was pumped in at the entry point and drawn out through the Porapak filter at the top. The design is based on Beale and colleagues (2006).

Eluted samples were stored at -20°C until further quantitative analysis using Gas Chromatography-Mass Spectrometry (GC-MS) (Section 2.8.2). The column was washed with 1 ml of diethyl ether three times before reuse; every wash was repeated only when diethyl ether had fully evaporated.

2.8.2 Coupled Gas Chromatography-Mass Spectrometry (GC-MS)

Quantitative analysis of volatile organic compounds was conducted using GC-MS. A Thermo Scientific TRACE 1310 Gas Chromatograph with a capillary GC column (Zebron ZB-1 50m x 0.32mm I.D., film thickness, Phenomenex) coupled with a Thermo Scientific ISQ^{TM} LT Single Quadrupole Mass Spectrometer was used. Ionization was by electron impact (70 eV, source temperature 250°C). The carrier gas, helium, was used at a constant flow rate of 2.6 ml/min. The oven temperature was maintained at 30°C for 5 minutes then programmed to increase the temperature to 230°C at 15°C/min. Total run time was 18.33 minutes. A volume of 1 µl per sample was injected into the column using a splitless injection. The inlet temperature was set at 200°C, the MS transfer line 240°C, and the ion source temperature 250°C. A split flow of 26ml/min was used and a splitless time of 0.8 minutes. MS mass range was 30-650 m/z with a dwell time of 0.35 seconds. Data was collected using Xcalibur software (Thermo Scientific). Principal Component Analysis (PCA) on the mass spectra was performed using MetaboAnalyst 2.0 using binned m/z and percentages of total ion count (%TIC) (Xia et al., 2015). Candidate compounds were identified by comparing their mass spectra with the mass spectra in the databases available in the Xcalibur software.

2.9 Statistics

Statistical analysis was carried out in R (R Core Team, 2017). The figures summarising continuous and discrete data are shown with mean ± standard error (SE). For experiments where choice tests were performed, a binomial function was used binom.test(). The nature of aphid-virus-host interaction experiments in this

project was mainly examined by count data and were analysed by general linear model (GLM), with a Poisson distribution (O'Hara and Kotze, 2010). For experiments with continuous data, ANOVA test or Student's *t*-test were performed. Details of each statistical test used are described in the results chapters for this thesis.

Chapter 3 Engineering CMV and TuMV resistance in transgenic plants

3.1 Introduction

In this Chapter, I describe the generation of CMV- and TuMV-resistant lines that I subsequently used in aphid transmission experiments (Chapter 5). Initially, I transformed plants of Arabidopsis accessions Col-0 and Ei-2 with a previously described amiRNA construct (ami-SD-3) (Duan et al., 2008). The effectiveness of CMV resistance obtained with this construct proved to be unsatisfactory. Better resistance was needed for my further experiments. Therefore, I designed two new constructs to generate independent CMV- and TuMV- resistant lines using a HP-RNAi vector (Sections 1.7.2 and 2.4). Expression of the two constructs was driven by the CaMV-derived 35S promoter and contained RNA sequences corresponding to the CMV *2b* sequence (HP-2b) and to the TuMV *P3N-PIPO* transcriptional slippage site (HP-PIPO), respectively (Section 1.3.2 and 1.4). Constructs were first tested by transient expression in *N. benthamiana* plants, and if satisfactory, then used to transform Arabidopsis and *N. benthamiana* for stable expression. I evaluated the degree of virus resistance by mechanical and aphid-mediated inoculation. For *N. benthamiana* transformed with HP-PIPO, I also evaluated the resistance to potyviruses other than TuMV. I performed aphid choice tests (Section 2.6.7) to evaluate whether the generated virus-resistant lines showed any alteration in their interactions with aphids (e.g. due to an effect of the transformation process or location of the T-DNA in the plant genome). This was done to avoid any artefacts of transformation affecting my subsequent virus-host-aphid interaction experiments (Chapter 5). Surprisingly, plants of one highly CMV-resistant line showed resistance to aphid settling. Therefore, differences between VOC emitted by this line and by non-transgenic Col-0 plants were also assessed.

3.2 Results

3.2.1 Optimisation of mechanical inoculation

To assess the degree of virus resistance, I needed to calibrate the virion concentration used for mechanical inoculation to choose a standardised concentration at which infection was 100% efficient. The concentrations tested on Arabidopsis plants (Col-0 and Ei-2) were 5,10, 20, 50 and 100 µg/ml for CMV and 5, 10, 20 and 50 µg/ml for TuMV. Table 3.1 shows the percentage of plants infected after mechanical inoculation with various virion concentrations (n = 20). The minimum virion concentration needed to achieve 100% inoculation efficiency on both Arabidopsis accessions was 20 µg/ml and 10 µg/ml for CMV and TuMV, respectively. TuMV virion concentrations at 5, 10 and 20 µg/ml were assessed on *N. benthamiana* plants (n = 12). As shown in Table 3.1, at all concentrations tested, inoculation efficiency was 100%. Hence, 5 µg/ml of TuMV virion was used to inoculate *N. benthamiana* plants routinely. Figure 3.1 shows representative examples of non-transgenic Arabidopsis and *N. benthamiana* plants 21 days after inoculation with CMV or TuMV at different concentrations.

3.2.2 Transient silencing of GFP-TuMV and GFP-2b by HP-PIPO and HP-2b constructs

Transient co-expression of the HP-PIPO construct with GFP-TuMV and the HP-2b construct with GFP-2b induced RNA silencing in *N. benthamiana* plants. Five days after co-infiltration, it was observed that only the areas where HP-empty vector was infiltrated with either GFP-2b or GFP-TuMV displayed fluorescence as shown in Figure 3.2. The absence of fluorescence in the zones where the HP-PIPO and HP-2b were infiltrated suggests that the constructs triggered RNA silencing.

Table 3.1 Optimisation of virus inoculum for mechanical inoculation

Host	Virus	Concentration (µl/ml)	% of plants infected
Arabidopsis (Col-0)	CMV	5	45%
		10	80%
		20	100%
		50	100%
		100	100%
	TuMV	5	65%
		10	100%
		20	100%
		50	100%
Arabidopsis (Ei-2)	CMV	5	40%
		10	45%
		20	100%
		50	100%
		100	100%
	TuMV	5	75%
		10	100%
		20	100%
		50	100%
N. benthamiana	TuMV	5	100%
		10	100%
		20	100%

Arabidopsis Col-0 and Ei-2 plants were inoculated with various virus concentrations in order to find the concentration at which inoculation efficiency was 100%.

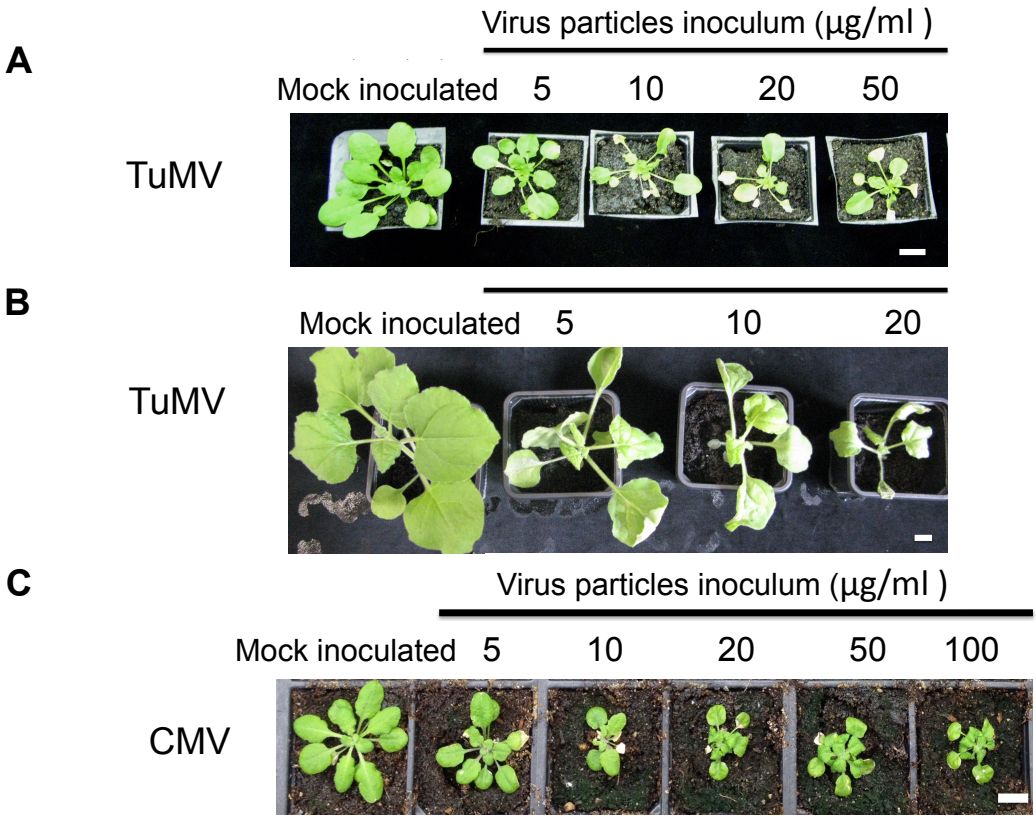


Figure 3.1 Infectivity assays to determine an effective virion concentration for mechanical inoculation

A shows Arabidopsis Ei-2 inoculated with TuMV virions at various concentrations (5, 10, 20 and 50 µg/ml). **B** shows *N. benthamiana* plants inoculated with three different concentrations of TuMV virions (5, 10, 20 µg/ml). **C** shows Arabidopsis Col-0 inoculated with CMV virions (5, 10, 20, 50, 100 µg/ml). All pictures were taken three weeks post-inoculation. Scale bar represents 1cm.

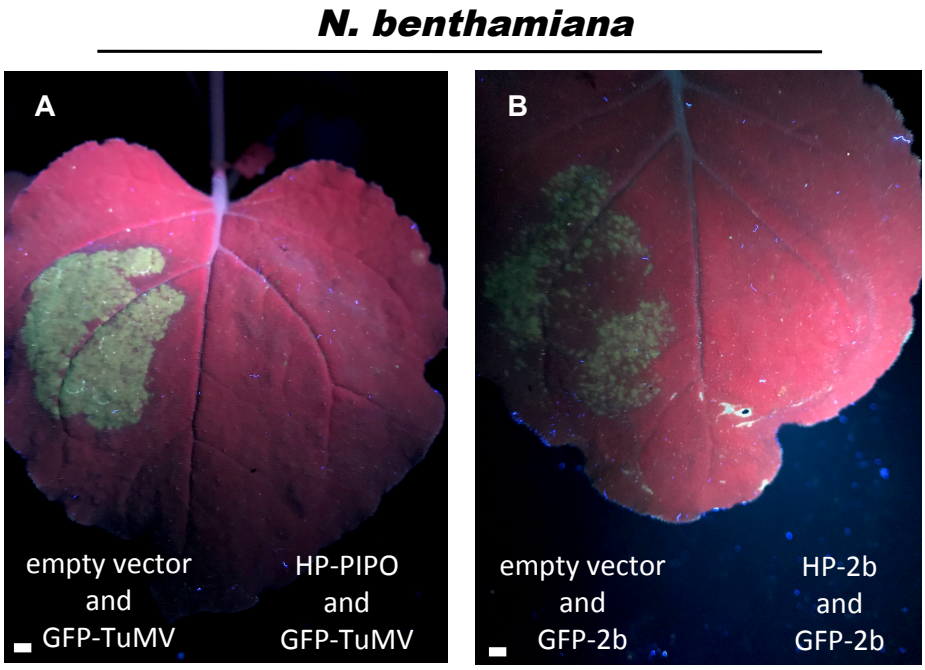


Figure 3.2 Transient expression in *N. benthamiana* to assess RNA silencing

A representative example of fully expanded *N. benthamiana* leaves of three-week old plants co-agroinfiltrated on the abaxial leaf surface. **A.** No fluorescence was observed on the right leaf patch where HP-PIPO and GFP-TuMV constructs were co-agroinfiltrated. The left patch was co-agroinfiltrated with the empty vector pK7GWIWG2(II)0 and GPF-TuMV and showed fluorescence five days after co-infiltration. **B** shows an infiltrated leaf where the left side shows fluorescence where the empty vector and GFP-2b constructs were co-infiltrated. In contrast, on the right patch where HP-2b and GFP-2b were co-infiltrated no fluorescence is observed.

3.2.3 Arabidopsis Col-0 and Ei-2 were transformed to confer resistance to CMV or TuMV

Plants of two Arabidopsis accessions (Col-0 and Ei-2) were transformed by floral dip transformation (Section 2.5.5) to generate virus-resistant plants for use in subsequent experiments (Chapter 5). The plants harbour constructs expressing one of the following transcripts: an ami-RNA to target a highly conserved region in the CMV genome; a HP-RNAi construct to target the CMV *2b* sequence, or a HP-RNAi construct to target the potyviral *P3N-PIPO* transcriptional slippage site (specifically, derived from TuMV (Section 2.4, Figure 2.1 and 2.2). Seeds of primary transformants (T1 generation) were collected from single plants transformed by floral dip transformation and germinated on media containing the appropriate antibiotic to select for seedlings expressing T-DNA inserts before transfer to soil and collection of T2 generation seeds (Sections 2.5.5 and 2.5.6). Once T1 or T2 plants were grown on soil and produced flower buds, the presence of the ami-RNA or RNAi sequences as well as hygromycin and kanamycin resistance genes were tested by PCR (Section 2.37) to confirm the presence of RNAi-inducing transgene sequences and antibiotic resistance gene sequences in each independent line (Figure 3.3). More transgenic lines were obtained in Arabidopsis accession Col-0 than in Ei-2 with all constructs (Table 3.2). I observed that the pattern of bolting and flowering differed between these two accessions (Figure 3.4) and that Ei-2 flowered at about two weeks later than Col-0. Thus, Ei-2 may have produced fewer flowers than Col-0 at the times plants were used for floral dipping.

Table 3.2 Primary transformants (T1) generated in Col-0 and Ei-2

Construct	Col-0	Ei-2
ami-SD-3	11	8
HP-2b	12	6
HP-PIPO	9	6

The number of primary transformants generated in Col-0 and Ei-2 are shown as well as the constructs used for floral dip transformation.

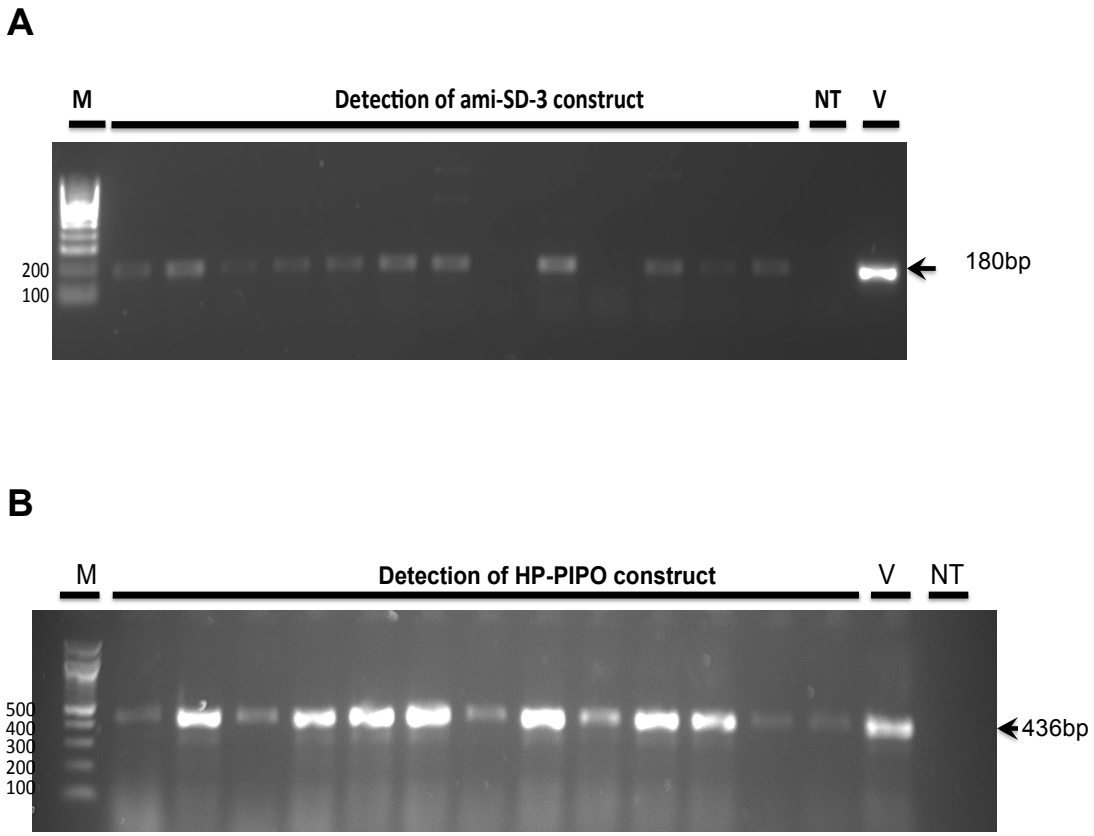


Figure 3.3 Example of PCR screening for presence of transgene in T2 generation transformed plants

Panel **A** shows expected bands amplified from T-DNA of the ami-SD-3 construct. Panel **B** shows amplified products corresponding to a specific region in the T-DNA containing HP-PIPO construct. Each lane represents an independent transgenic plant. PCR products were separated in 1.5% (w/v) agarose gels in 1X TAE buffer. Marker (M) lanes were loaded with Hyperladder 100 bp (Bioline) DNA size markers. DNA extracted from non-transgenic (NT) plants were used as negative controls. Plasmid vectors (V) carrying the two constructs were used as positive controls.

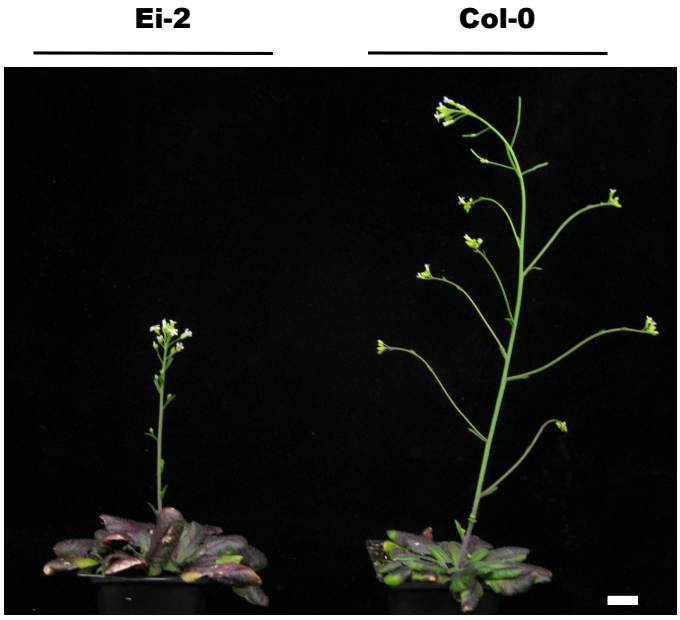


Figure 3.4 Flowering phenotypes of Ei-2 and Col-0 plants
It was noted that Ei-2 had a late flowering time compared with Col-0. Picture was taken 8 weeks post-germination. Scale bar represents 1 cm.

3.2.4 Transformation of *N. benthamiana* with HP-PIPO construct

N. benthamiana plants were transformed with the HP-PIPO construct (Section 2.5.6). It was hypothesised that transgenic lines expressing the highly conserved *P3N-PIPO* transcriptional slippage sequence (Section 1.4) would be resistant to a wide range of potyviruses.

A total of 13 independent lines were generated. All transgenic lines showed the presence of transgene by PCR and displayed a similar wild-type phenotype. The plants generated were challenged with TuMV and two other potyviruses, potato virus Y (PVY) and bean common mosaic virus (BCMV). T2 generation plants were assessed by mechanical inoculation and aphid-inoculation with GPF-TuMV. As shown in Figure 3.5, HP-PIPO line W2(4) challenged by mechanical inoculation with TuMV was fully resistant to the virus as the plant did not show any viral symptoms. Ten 7-day-old aphids were moved individually from systemically-infected GFP-TuMV plants to TuMV-resistant plants. The site of aphid-inoculation was noticed and the inhibition of virus spread was detected (Figure 3.7). The HP-PIPO construct did not confer resistance to other potyviruses evaluated, PVY and BCMNV, as the inoculated plants with either virus show similar symptoms compared with the non-transgenic *N. benthamiana* (Figure 3.6).

3.2.5 Assessing virus resistance of transgenic plants by mechanical inoculation or aphid-inoculation

Virus-resistant plants harbouring either the ami-SD-3, the HP-PIPO or the HP-2b construct in Arabidopsis Col-0 and Ei-2 backgrounds were challenged with viruses using either mechanical inoculation or aphid-inoculation (Section 2.5.8). An example of a CMV-resistant (Col-0 HP-2b 2.4.2) line exhibiting strong resistance to virus infection delivered by mechanical and aphid-mediated inoculation is shown in Figure 3.8.

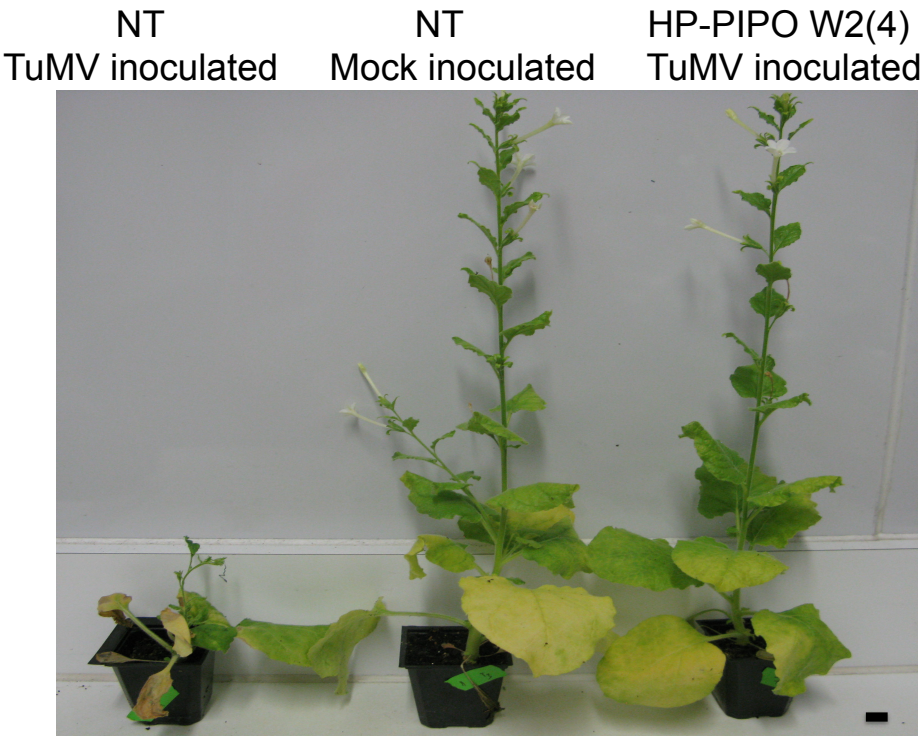
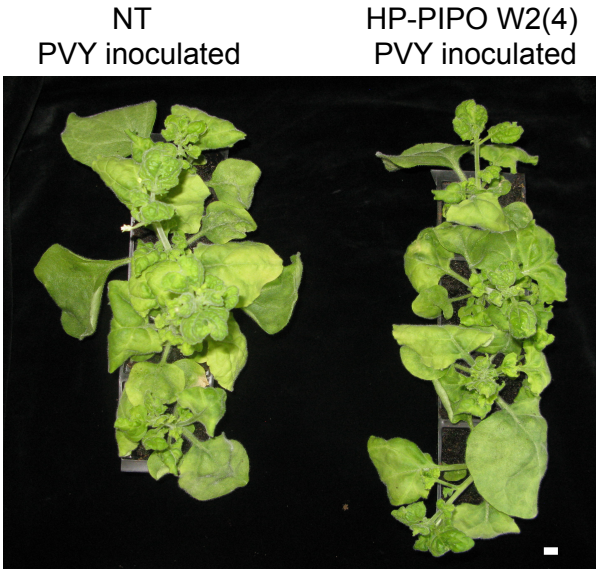


Figure 3.5 Assessment of resistance to TuMV in plants expressing the HP-PIPO construct
N. benthamiana plants were inoculated with TuMV three weeks after germination. The plant on the **left**, NT, is a non-transgenic plant that shows TuMV symptoms. The plant in the **middle** is a non-transgenic mock-inoculated plant. The plant on **right** side is a HP-PIPO line that exhibits complete resistance to TuMV. Pictures were taken after six weeks of inoculation. Scale bar = 1cm.

A



B

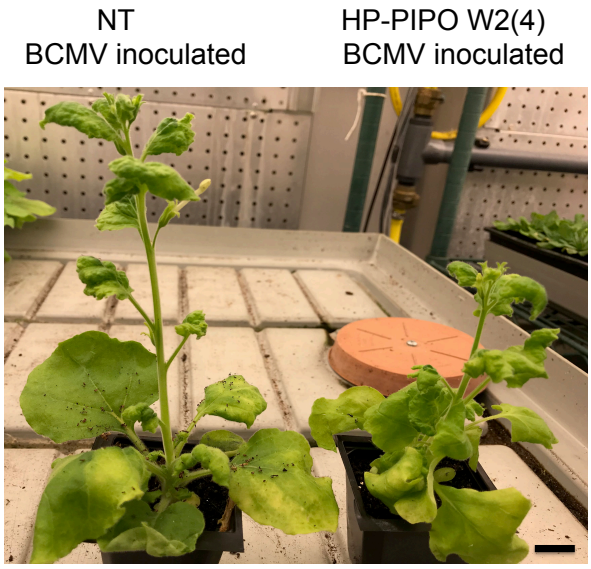


Figure 3.6 Assessment of HP-PIPO construct against PVY and BCMNV

N. benthamiana non-transgenic and HP-PIPO plants were inoculated with PVY (n =7) and BCMNV (n = 7) three weeks after germination. **A** shows non-transgenic (NT) and the line HP-PIPO W2(4) after three weeks post-inoculation. PVY spread systemically in both transgenic and non-transgenic plants. **B** exhibits plants inoculated with BCMV and also show systemic spread of the virus. The experiment was repeated two times with similar results. Pictures were taken five weeks post-inoculation. Scale bar = 1cm.

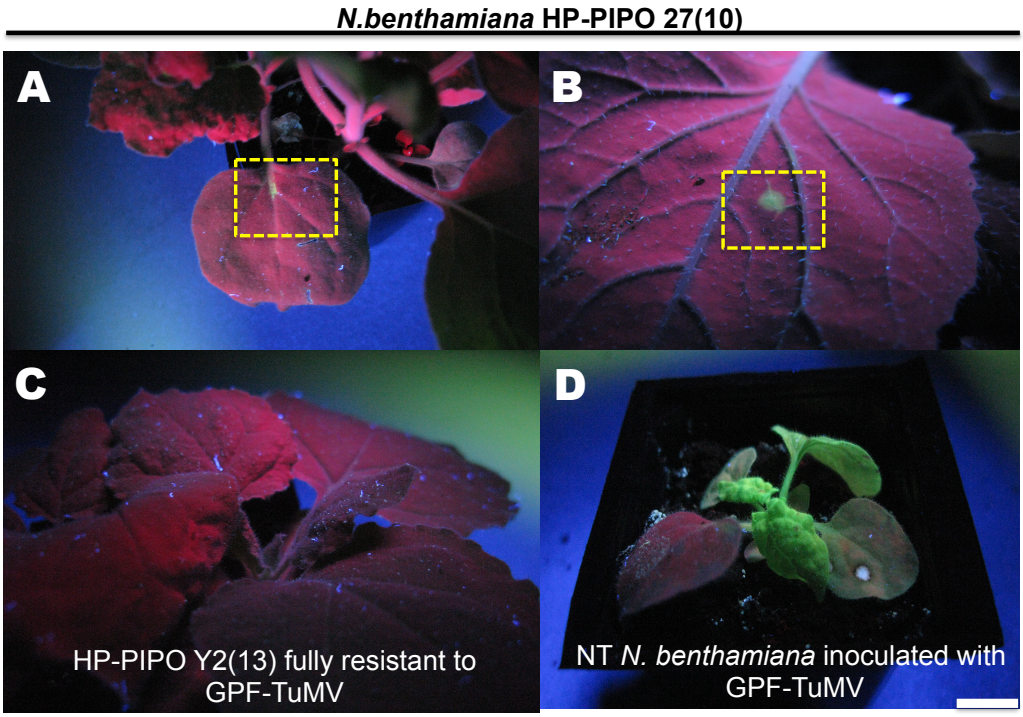


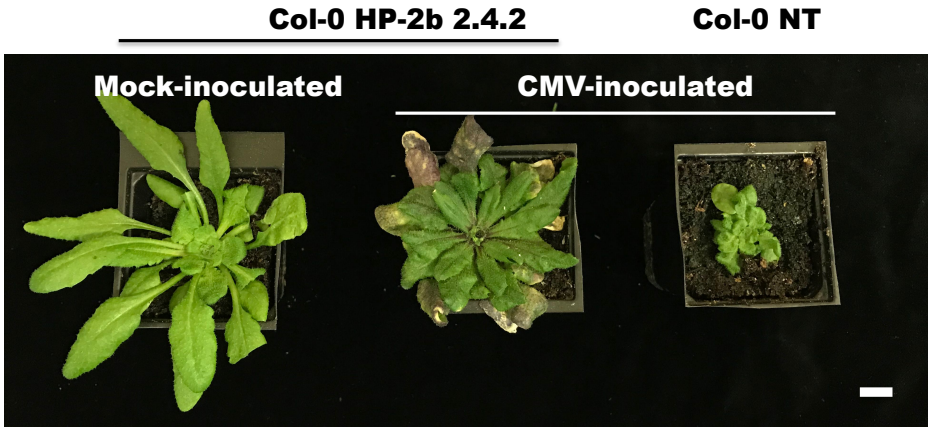
Figure 3.7 HP-PIPO *N. benthamiana* lines are fully resistant to TuMV
HP-PIPO *N.benthamiana* lines were challenged with GFP-TuMV by aphid-inoculation. Panel **A-B** display line 27(10) on which aphid-inoculation sites (yellow rectangle) were detected. Panel **C** exhibits Y2(13) line that is fully resistant to TuMV since no GFP signal is even observable at aphid-inoculation sites. Panel **D** shows a non-transgenic plant (NT) systemically-infected with GFP-TUMV. All pictures were taken three weeks post-inoculation. Scale bar represents 1 cm.

Virus-resistant plants showing no symptoms or mild symptoms were also evaluated for virus accumulation (Section 2.2.12). Virus titres on both CMV- and TuMV-resistant plants were significantly lower than virus-infected non-transgenic plants (Figure 3.9) (Student's *t* test, $p < 0.001$). Table 3.3 shows the selected lines for further experiments in Chapter 5. It can be noted that the proportion of resistant plants seen by mechanical and aphid-inoculation were similar in the range of 70%-100%. An overall trend was observed that virus-resistant plants showed virus resistance equal to or above 70% (Table 3.3). Particularly, the line HP-PIPO line 5.53 in Col-0 background showed complete resistance to TuMV as shown by ELISA (Figure 3.9). A detailed list of all Arabidopsis and *N. benthamiana* transgenic lines assessed for virus resistance by mechanical and aphid inoculation is shown in Appendix 3. A list of lines harbouring ami-SD-3 evaluated by mechanical inoculation is shown in Appendix 4. The lines shown in Appendix 4 were not evaluated by aphid inoculation because these lines show a virus resistance below 50%.

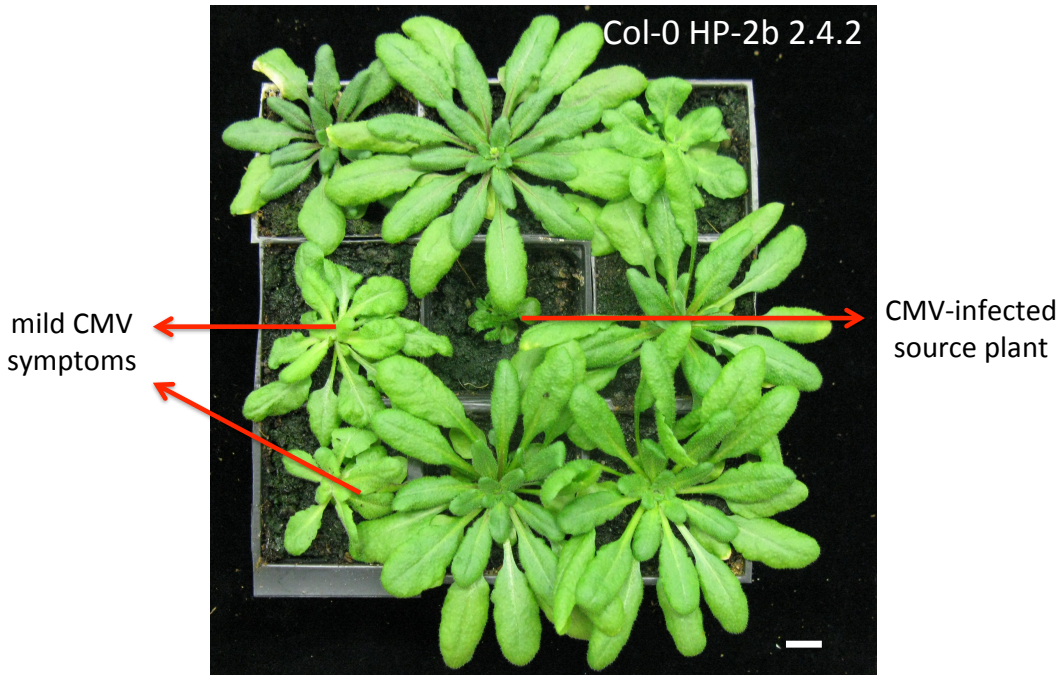
3.2.6 The phenotypes of virus-resistant transgenic plants

Plants of virus-resistant lines were grown alongside non-transgenic plants under long day conditions and no differences in growth or flowering were observed (Figure 3.10). All lines showing resistance to CMV or TuMV were subjected to free two-way choice aphid settling assays (Section 2.6.7) to ensure that transformation had not affected genes controlling plant-aphid interactions. In most cases (Figure 3.11 and Table 3.3), transformation had no effect on this. As shown in Figure 3.11 the transgenic lines Col-0 ami-SD-3 5.17, Ei-2 HP-2b 1.13 and Col-0 HP-PIPO 5.53 exhibited similar degree of aphid settlement on transgenic and non-transgenic plants after 1 hour and 24 hours of aphid release. But in one case (line Col-0 ami-SD-3 1.12) host-aphid interaction was affected because aphids were reluctant to settle on line Col-0 ami-SD-3 (Figure 3.12, Section 3.27).

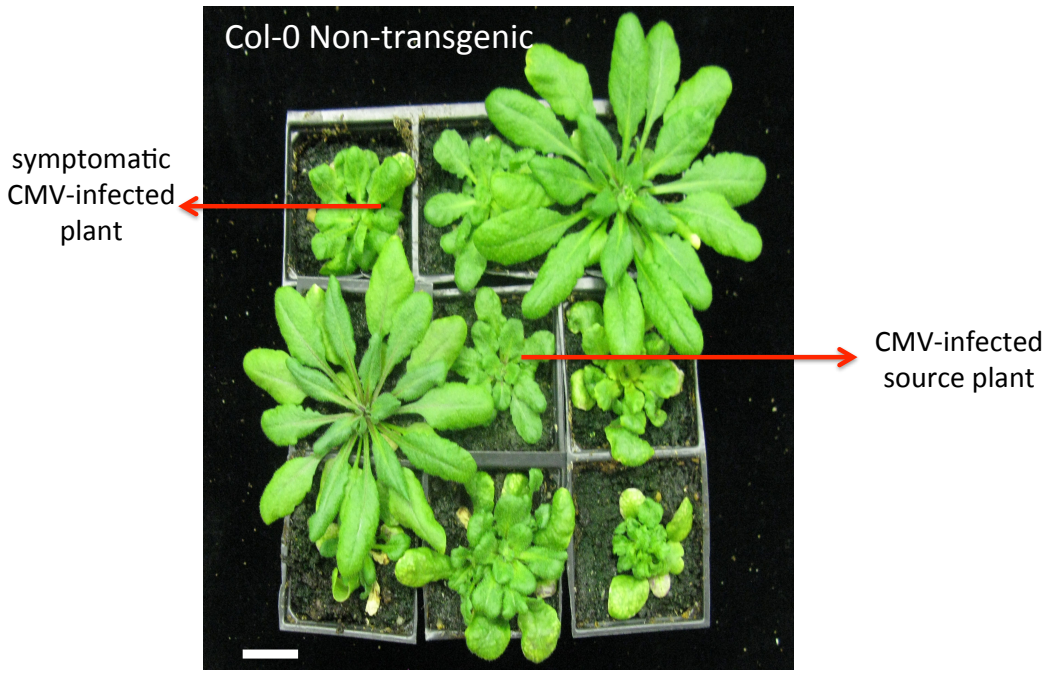
A **Mechanical inoculation**



B **Aphid inoculation**



C **Aphid inoculation**



D **Mock-inoculated (control)**

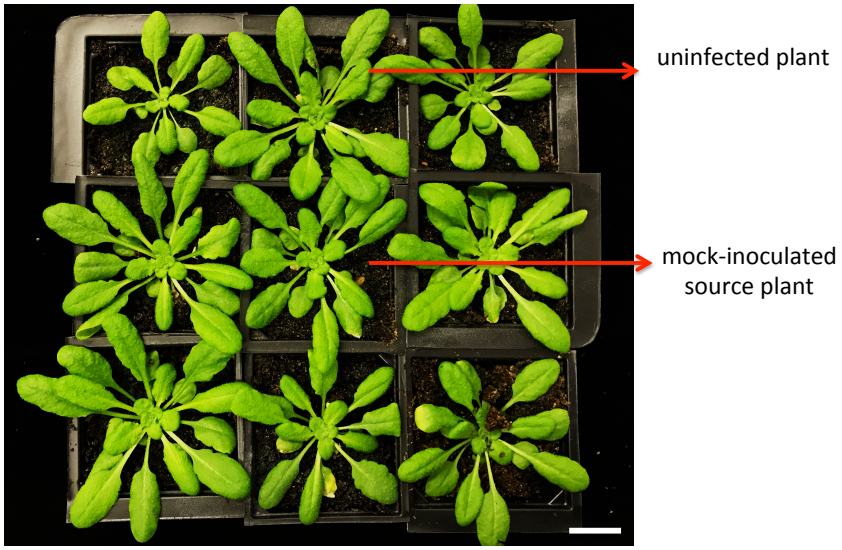


Figure 3.8 Example of a transgenic CMV-resistant line challenged by mechanical and aphid-inoculation
A shows Col-0 HP-2b-2.4.2 transgenic line that exhibits moderate symptoms compared to non-transgenic (NT) plants challenged with CMV (20 µg/ml). **B-D** display 3x3 arrays used for aphid-inoculation. **B** CMV-infected source plants are placed in the centre and are surrounded by Col-0 HP-2b-2.4.2 or **C** Col-0 non-transgenic plants. **D** shows an array were the source plant was mock-inoculated and no virus transmission was observed. Scale bars represent 1 cm.

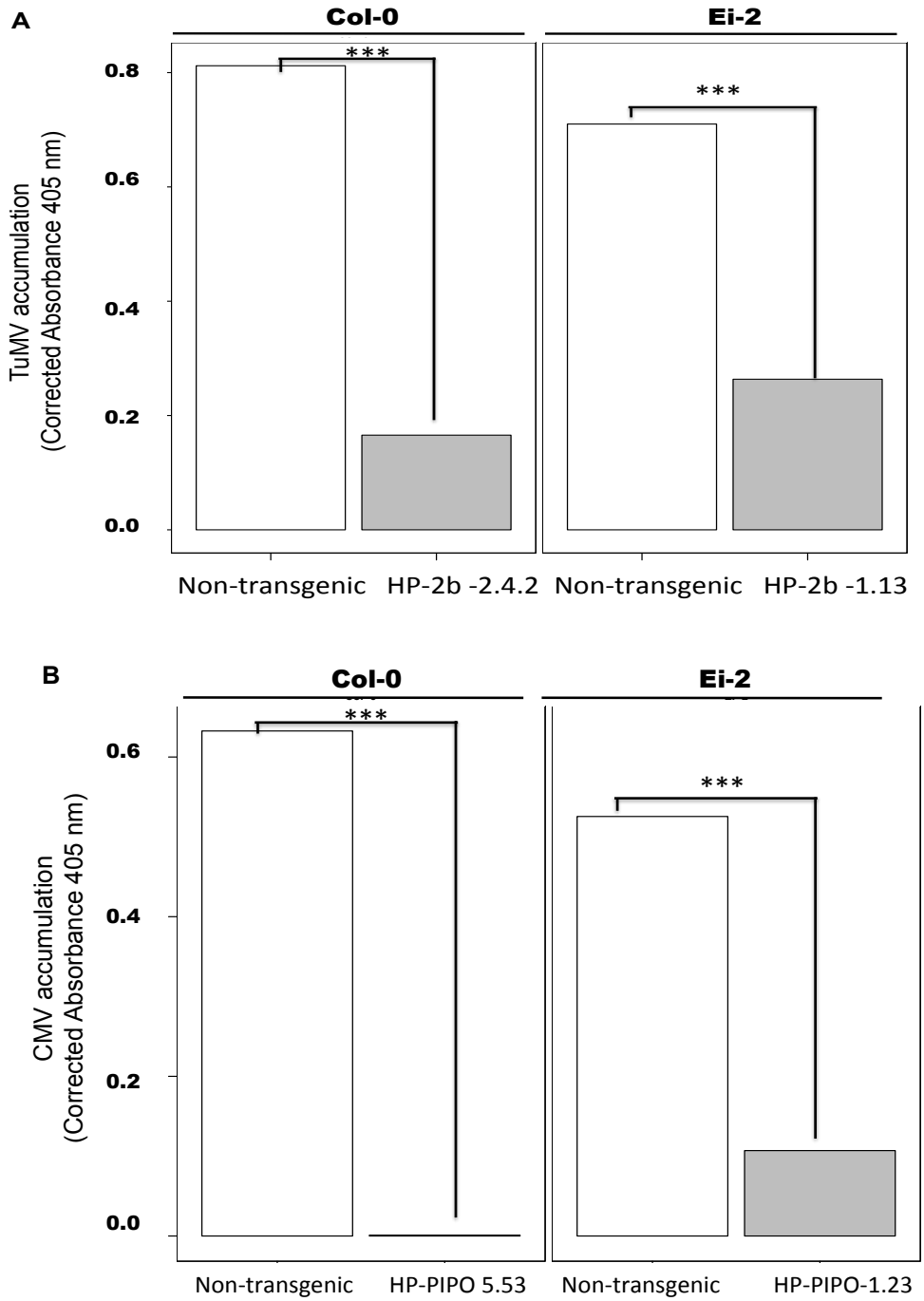


Figure 3.9 Virus-resistant plants accumulate less virus than non-transgenic plants

HP-2b and HP-PIPO independent lines were inoculated with CMV and TuMV, respectively. Plants showed no symptoms or mild symptoms were analysed by ELISA. Less virus accumulation was detected in transgenic plants compared with non-transgenic plants. The values of blanks and values of samples of mock-inoculated plants were subtracted to obtain the corrected absorbance values displayed. **A** shows CMV concentration in Col-0 and Ei-2 CMV-resistant plants compared with non-transgenic plants. **B** shows TuMV concentration in Col-0 and Ei-2 TuMV-resistant plants compared with non-transgenic plants.

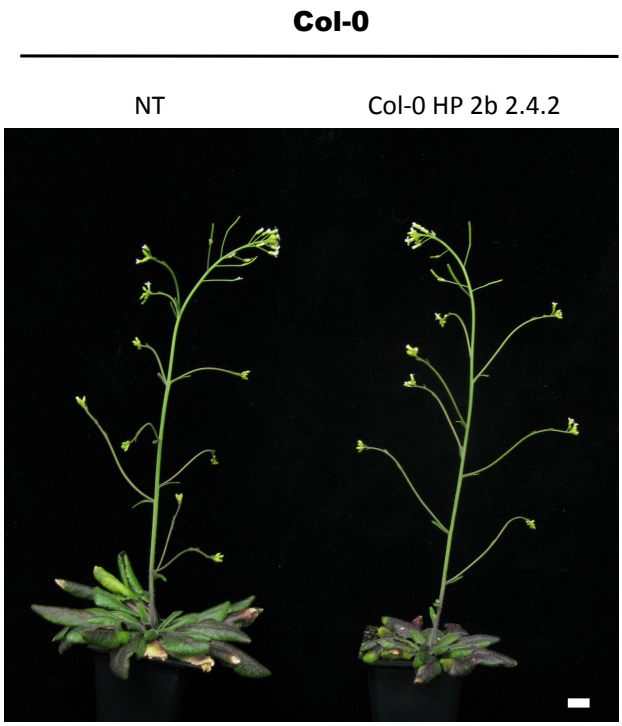


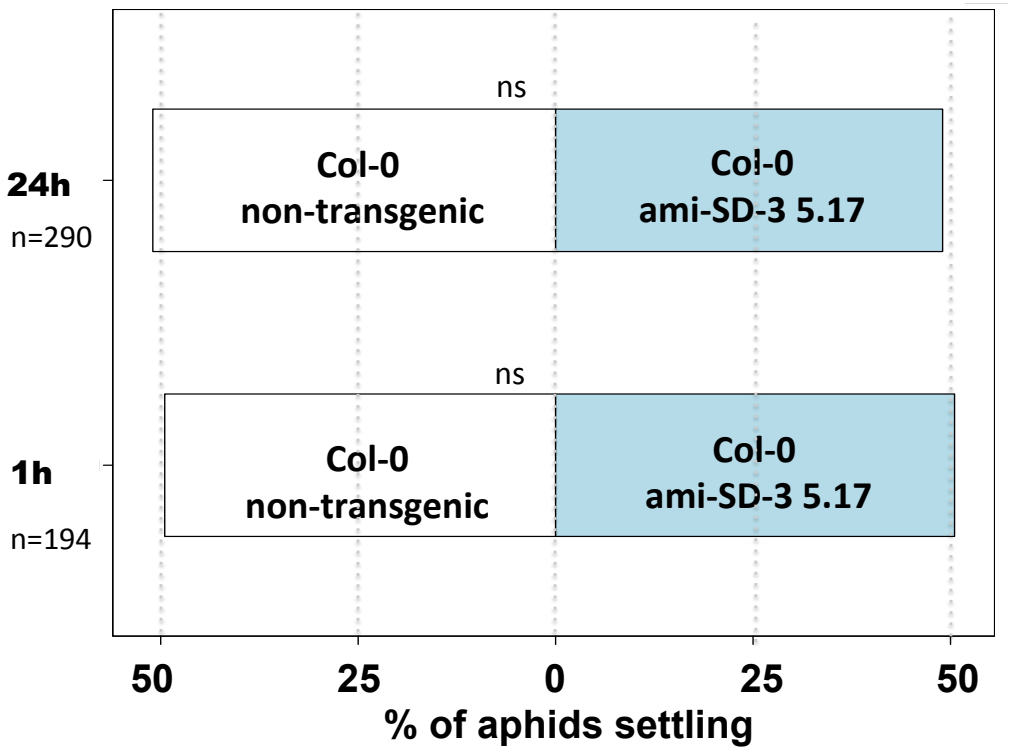
Figure 3.10 Transgenic plants showed no change in flowering phenotype
A plant of Col-0 HP-2b 2.4.2 line exhibited similar growth and development as a non-transgenic Col-0 plant. Scale bar represents 1 cm.

Table 3.3 List of virus-resistant lines generated in Arabidopsis accessions

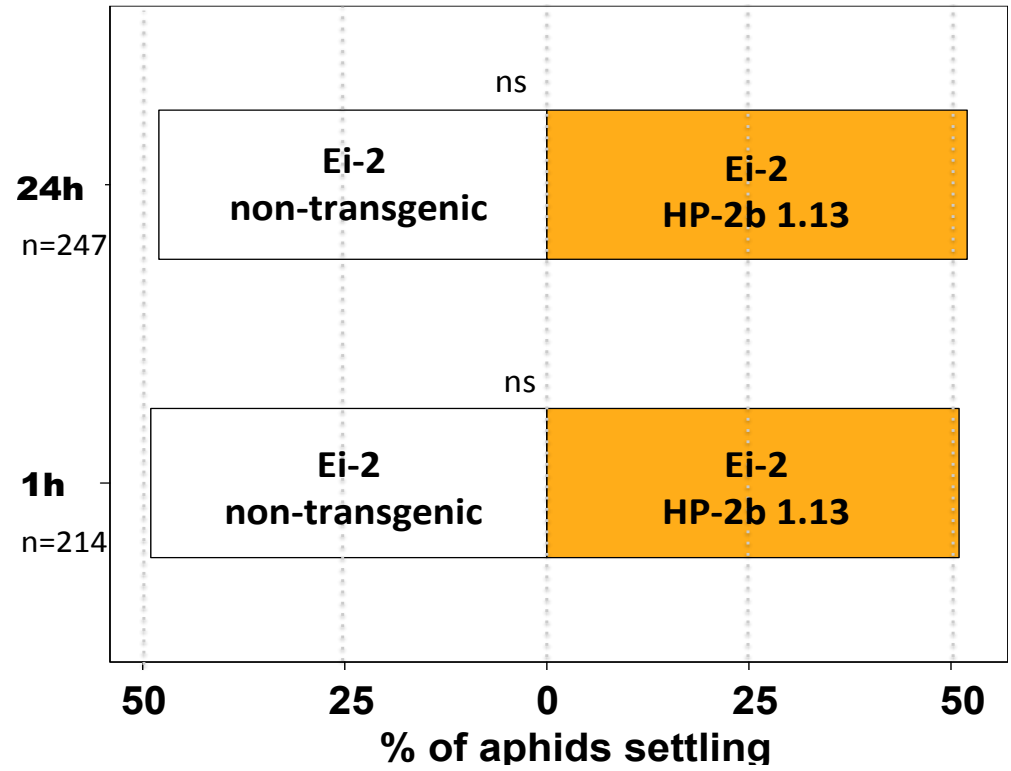
Accession	Line	Construct	Virus tested	Proportion of resistant plants		Chi-squared	Aphid repellent or attractive
				Mechanical inoculation	Aphid inoculation	p value	
Col-0	**5.17**	SD-3	CMV	80%	75%	0.71	No
	1.12	SD-3	CMV	80%	70%	0.5	Yes
	2.4.2	HP-2b	CMV	94%	91%	0.72	No
	5.53	HP-PIPO	TuMV	100%	100%	1	No
Ei-2	**7.1**	SD-3	CMV	80%	88%	0.49	No
	1.13	HP-2b	CMV	87%	83%	0.73	No
	1.23	HP-PIPO	TuMV	73%	75%	0.89	No

The virus-resistant lines shown in the table are the transgenic lines in Col-0 and Ei-2 backgrounds that exhibited the highest or complete resistance to the virus tested by mechanical and aphid-inoculation. It is also shown whether the lines were aphid deterrent. A complete list of transgenic plants evaluated and the number of plants evaluated per construct is given in Appendix 3 and 4.

A


ns
Col-0
non-transgenic
Col-0
ami-SD-3 5.17
24h
n=290
ns
Col-0
non-transgenic
Col-0
ami-SD-3 5.17
1h
n=194
50
25
0
25
50
% of aphids settling

B


ns
Ei-2
non-transgenic
Ei-2
HP-2b 1.13
24h
n=247
ns
Ei-2
non-transgenic
Ei-2
HP-2b 1.13
1h
n=214
50
25
0
25
50
% of aphids settling

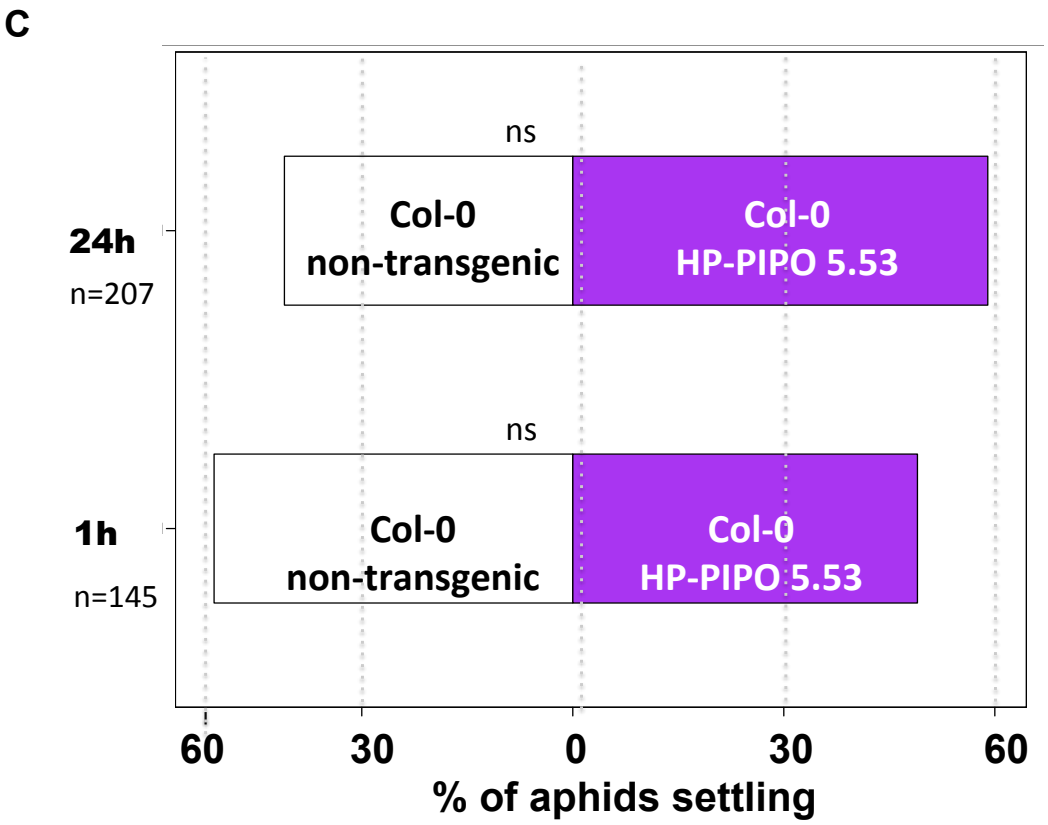


Figure 3.11 Transgenic and non-transgenic plants showed same degree of aphid attractiveness
Thirty aphids were released in pot arenas containing virus-resistant transgenic plants and non-transgenic plants. At 1 hour and 24 hours after release, aphids on each plant were counted. Ten pots were assessed per treatment. **A** Col-0 ami-SD-3 5.17, **B** Ei-2 HP 2b 1.13 and **C** Col-0 HP-PIPO 5.53. Aphids had no preference between virus-resistant plants and non-transgenic plants. No statistical significance differences were found based on binomial tests. Only aphids found (n) on the plants were used for analysis.

3.2.7 Col-0 ami-SD-3 1.12: A highly CMV-resistant plant that is also not suitable for aphid settlement

Aphids were reluctant to settle on one CMV-resistant transgenic line (Col-0 ami-SD-3 1.12) (Figure 3.12 and Table 3.3). At 1 hour after aphid release, 39% of aphids settled on Col-0 ami-SD-3 1.12, whereas at 24 hours only 12% of aphids settled on the transgenic line (binomial test, $p < 0.001$). To further investigate the observed aphid reluctance to settle on Col-0 ami-SD-3 1.12 plants, I analysed the emission of VOCs and the performance of aphids confined on plants of this line (Sections 2.5.1, 2.5.2 and 2.7).

I trapped volatiles emitted by Col-0 non-transgenic and Col-0 ami-SD-3 1.12 plants and analysed them by GC coupled to MS (Figure 3.13). The emitted VOCs were distinct from each other when compared by principal component analysis on the relative abundance of ions (over 75 Da) within the samples (Figure 3.13). PC1 explained most (97%) of the variation between non-transgenic plants and Col-0 ami-SD-3 1.12 plants. This shows there are qualitative differences in the total blend of volatiles emitted by these plants, which may affect aphid preferences.

I attempted to identify the semiochemicals that differed between Col-0 ami-SD-3 1.12 from non-transgenic Col-0 plants. I found one putative candidate that differs between the VOCs from Col-0 ami-SD-3 1.12 transgenic plants and the VOCs from non-transgenic plants (Figure 3.14).

I also evaluated whether the concentration of four volatiles that I identified in Col-0 plants, as 3-methyl-hexane, (*E*)-2-hexene-1-ol, 2-octanone and 3-pentanol (Chapter 4, Figure 4.12) were different between the transgenic line Col-0 ami-SD-3 1.12 and Col-0 non-transgenic. I found no differences in the combined concentration of these volatiles (Figure 3.15). The results indicate that emission of 3-methyl-hexane, (*E*)-2-

hexene-1-ol, 2-octanone and 3-pentanol were not altered in the transgenic line Col-0 ami-SD-3 1.12.

Col-0-ami-SD-3 1.12 plants were compared with non-transgenic plants and plants of another CMV-resistant line, Col-0 ami-SD-3 5.17 that did not show negative aphid settlement (Figure 3.11 and Table 3.3) in their ability to support aphid growth. MRGR experiments showed that aphid growth on Col-0 ami-SD-3 1.12 (0.35 ± 0.02 mg/mg/day) was not statistically different to non-transgenic plants (0.37 ± 0.01 mg/mg/day) and the transgenic line Col-0 ami-SD-3 5.15 (0.39 ± 0.02 mg/mg/day) (Figure 4.16A). However, colony size (number of nymphs) on Col-0 ami-SD-3 1.12 was significantly lower (2.3 ± 0.6) compared with the colony size on the non-transgenic plants (4.7 ± 0.7) and colony size on the Col-0 ami-SD-3 5.17 plants (5.3 ± 1.0) (One-way ANOVA, $p < 0.05$) (Figure 4.16B). Collectively, these results indicate that the CMV-resistant line Col-0 ami-SD-3 1.12 is deterrent to aphids due to its distinct VOC profile and it is also a less suitable host for aphids.

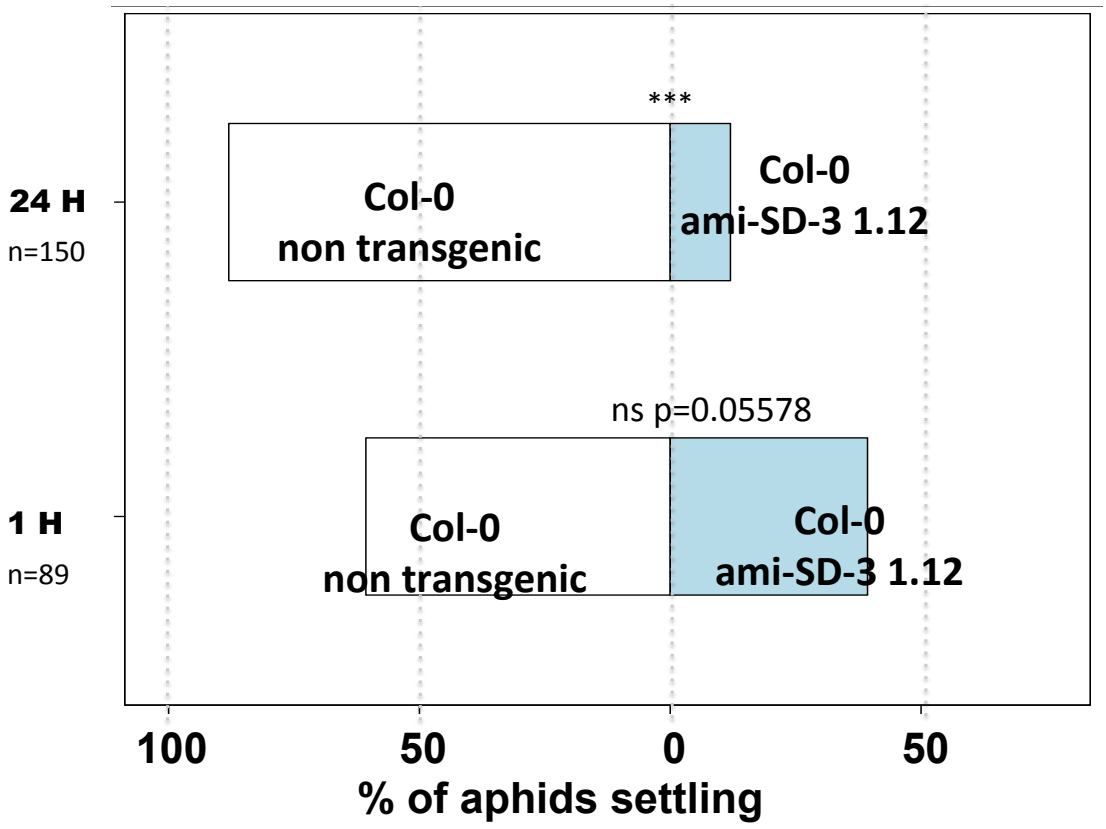


Figure 3.12 Plants of the CMV-resistant Col-0 ami-SD-3 line 1.12 repel aphids

Aphid settling preference choice tests were performed between Col-0 non-transgenic plants (NT) and ami-SD-3 1.12 transgenic line. After 1 hour and 24 hours of aphid release, aphids settled on Col-0 non-transgenic plants. The ami-SD-3 1.12 line repelled aphids as only 12% chose to settle on Col-0 ami-SD-3 1.12. Statistical differences were analysed by binomial test, $p < 0.001$ (***). Only aphids found (n) on the plants were used for analysis.

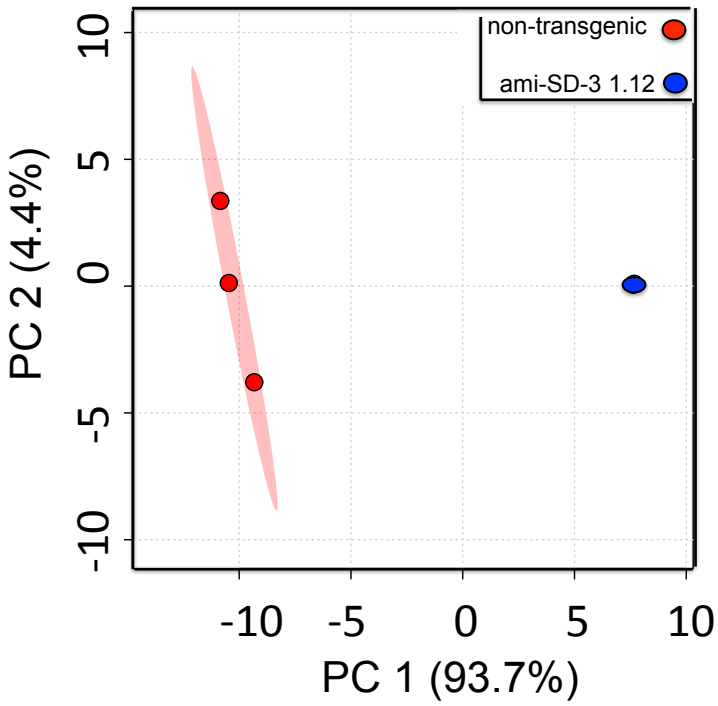


Figure 3.13 VOCs of Col-0 ami-SD-3 1.12 differ from those emitted by non-transgenic Col-0 plants

Principal component analysis of m/z values (over 75 Da) obtained by gas chromatography-mass spectrometry of volatile organic compounds collected from non-transgenic (n = 3 pots containing 20 plants) and transgenic line ami-SD-3 1.12 (n = 3 pots containing 20 plants) plants. Col-0 ami-SD-3 1.12 samples are clustered distinctly from non-transgenic plants. The percentage of variation that is explained by PC1 and PC2 was 93.7% and 4.4%, respectively.

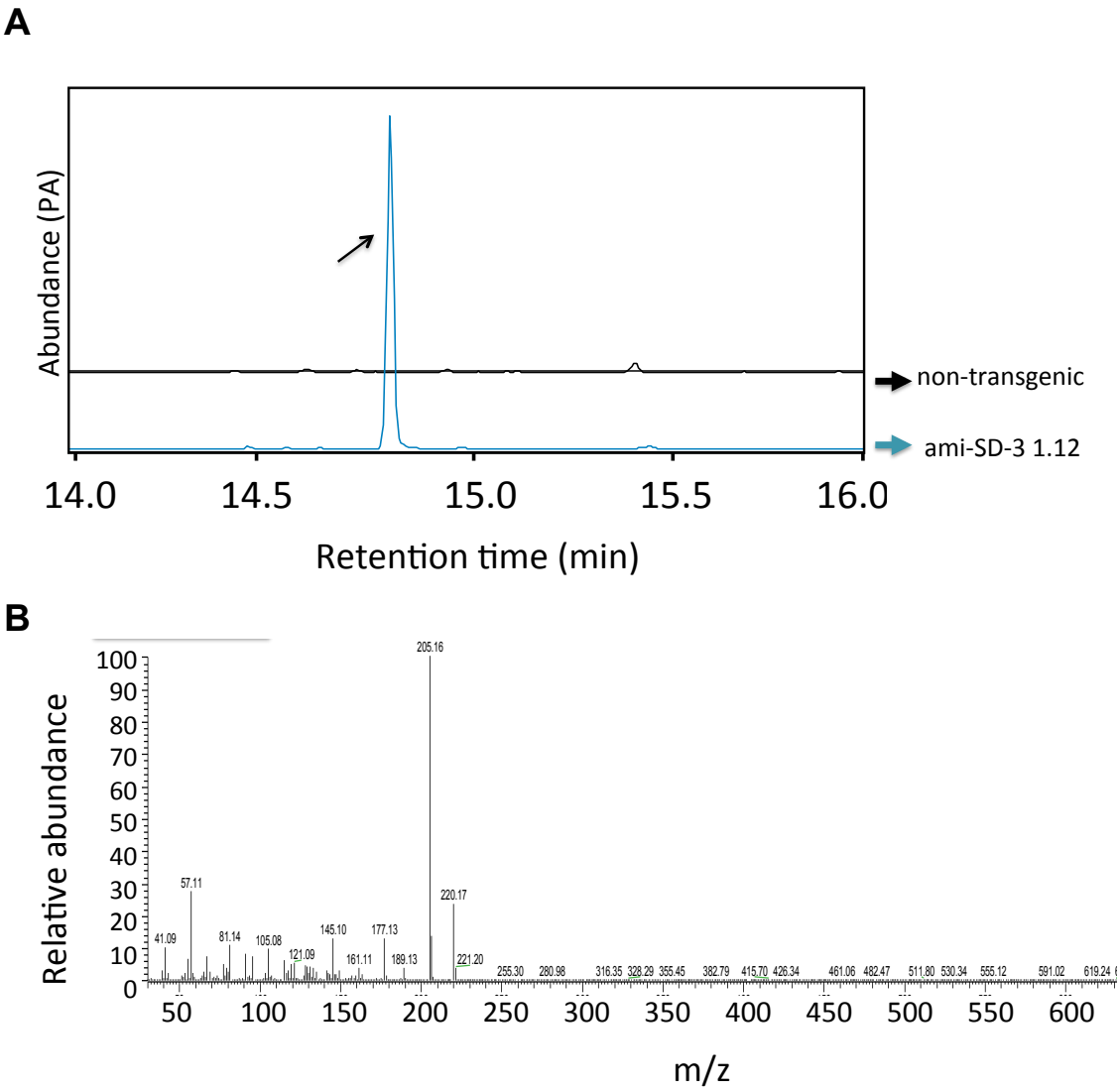


Figure 3.14 Gas chromatography-mass spectra profile of VOCs emitted by transgenic Col-0 ami-SD-3 1.12 plants

A Representative gas chromatograms of eluted peaks from VOCs samples from Col-0 ami-SD-3 1.12 and non-transgenic Col-0 plants were overlayed to identify different peaks between the samples (n = 3). A putative candidate at a retention time of 14.8 minutes shows a relatively higher abundance in the VOCs sample from the transgenic plant ami-SD-3 1.12 than in the VOCs sample from non-transgenic plants. **B** Mass spectrum of the putative candidate that was present in the chromatograms from VOCs samples from Col-0 ami-SD-3 1.12 but not observed in the chromatograms from VOCs samples from non-transgenic Col-0 plants.

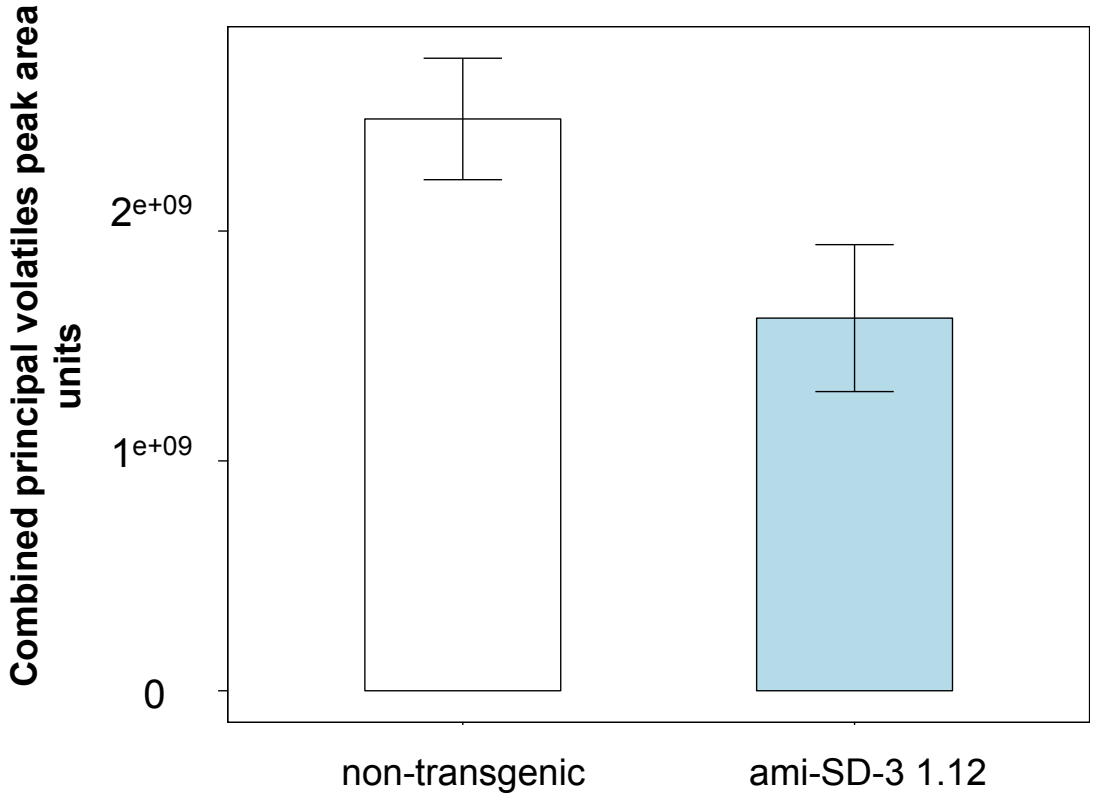


Figure 3.15 Concentrations of volatiles identified in Arabidopsis are not altered in Col-0 ami-SD-3 1.12 plants

The combined peak areas of 3-methyl-hexane, (*E*)-2-hexene-1-ol, 2-octanone and 3-pentanol found in Arabidopsis plants (Chapter 4, Figure 4.13) were compared with transgenic line Col-0 ami-SD-3 1.12. The individual peak areas of these four compounds were not significantly different from the non-transgenic sample (Student's *t* test).

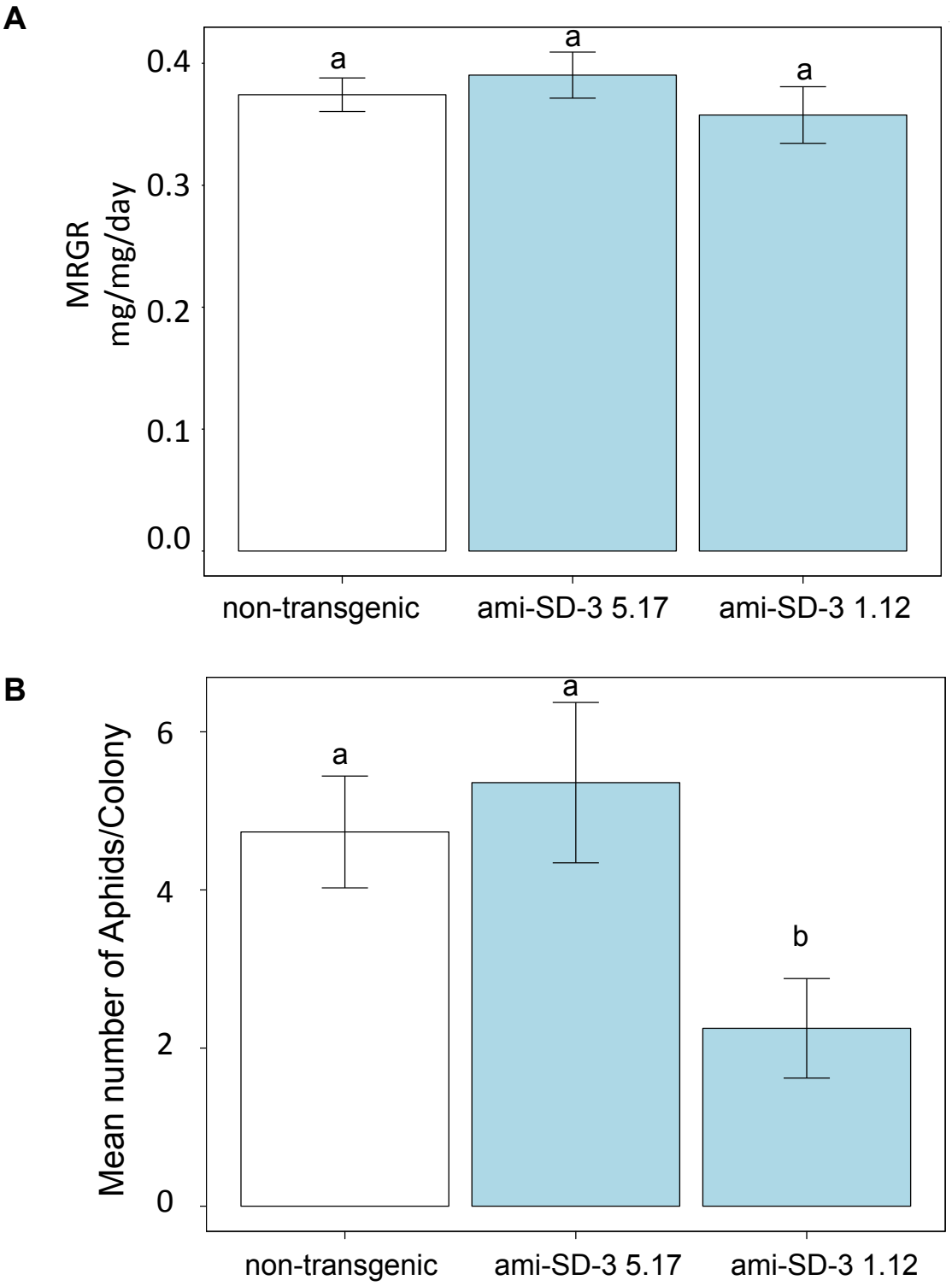


Figure 3.16 CMV-resistant Col-0 ami-SD-3 line 1.12 affects aphid reproduction

Panel **A** shows the results of a mean relative growth rate (MRGR, mg/mg/day) assay. There was no significant difference between plants evaluated, n = 15. Panel **B** indicates the result of a colony growth assay. Aphid reproduction was decreased on Col-0 ami-SD-3 1.12 compared with aphid growth on non-transgenic plants and on ami-SD-3-5.17 plants n = 15. One-way ANOVA, Tukey post hoc test, plants not sharing the same letter are significantly different to the control (non-transgenic plants), $p < 0.05$.

3.3 Discussion

3.3.1 Generation of virus-resistant Arabidopsis lines

The type of virus strain used to challenge transgenic plants expressing the ami-SD-3 construct of Duan et al. (2008) may explain the weaker resistance observed in CMV-resistant ami-SD-3 Arabidopsis plants. Duan and colleagues (2008) challenged CMV-resistant plants expressing ami-SD-3 construct with sap inoculum of SD-CMV strain, which belongs to subgroup IB. They reported 100% resistance in Arabidopsis Col-0 plants expressing the ami-SD-3 construct. In contrast, I challenged the ami-SD-3 lines with a subgroup IA strain, Fny-CMV virions (20 µg/ml). Thus, a different strain and source of inoculum may give different results when assessing the degree of virus-resistant plants. An alternative explanation would be that Duan et al. (2008) chose the amiRNA target site based on the abundance of virus-derived siRNAs generated from this section of the CMV sequence. At the time it was thought that such a sequence might be an Achilles' heel that was sensitive to degradation by the host silencing machinery. Nowadays, it is recognized that virus-derived siRNAs may act as pathogenesis factors (Shimura et al., 2011; Smith et al., 2011). Hence, attacking a region that is a hot spot for siRNAs with amiRNAs may be less effective for resistance generations than previously thought.

3.3.2 The selection of target sequences is crucial for effective RNAi-based resistance

The results suggest that the CMV *2b* and TuMV *P3N-PIPO* sequences are good targets to use for selection of RNA inducing sequences as resistance-inducing transgenes. Although transient assays for the HP-2b construct showed promising results, the stable transformants were more variable in the degree of virus resistance. Different locations of T-DNA insertion into the plant genome of transgenic plants might also explain the variability as transgenic lines are always different from each other (Kaniewski and Thomas, 1999).

The HP-PIPO construct was highly efficient in inducing virus resistance against TuMV which may be a result of the reduction of its function. The P3N-PIPO protein is involved in viral cell-to-cell movement (Wei et al., 2010). Although GAAAAAA transcriptional slippage sequence is highly conserved among potyviruses, the sequence is only 4-6 nucleotides long and the flanking regions may not be similar enough in different potyviruses to induce broad resistance (Olspert et al., 2015). This likely explains why *N. benthamiana* transgenic plants were resistant to TuMV but not to two other potyviruses, PVY and BCMV.

3.3.3 Mechanical and aphid-inoculation are critical to assessing virus resistance

The importance of evaluating the level of resistance using both mechanical and aphid-inoculation was critical to determine if the virus-resistant plants generated were still resistant under aphid-inoculation pressure. The results indicate that virus-resistant plants showed a similar degree of resistance by mechanical and aphid-inoculation. I could not detect significant differences between the types of inoculation. The high number of aphids used for inoculation might have contributed to the similar results obtained with mechanical inoculation. The results showed that virus-resistant plants expressing the constructs ami-SD-3, HP-2b and HP-PIPO displayed a reduction in the accumulation of virus. These results clearly suggest that the high degree of resistance observed is related to a reduction in virus accumulation due to inhibition of virus replication.

3.3.4 A single CMV-resistant line affected aphid behaviour: an effect of T-DNA location?

All CMV- and TuMV-resistant lines obtained were identical to non-transgenic plants in terms of growth and development. Because the lines were going to be used in

further aphid behaviour assays (Chapter 5), I decided to test whether the generated lines had any effect on aphid settling assays. Only one of the transgenic line exhibited alterations in plant-aphid interactions. Aphids were reluctant to settle on the CMV-resistant line Col-0 ami-SD-3-1.12. I evaluated the ami-SD-3 line for effects on the growth and reproduction of aphids confined on plants of this line and investigated VOC emission of this line. I found that the VOC blend emitted by plants of this transgenic line was different from non-transgenic Col-0 plants. Although the growth rate of individual aphids placed on plants of this line was not changed, aphid reproduction was. Presumably, this unexpected phenotype is explained by the location of the T-DNA. Potentially, the T-DNA landed in a genomic region where a gene involved in aphid defence is located. It has been suggested that T-DNA insertion may lead to different possible outcomes depending on the place it landed (Krysan et al., 1999). I attempted to identify the region sequence where the T-DNA landed using walking PCR, but there was insufficient time to complete this effort.

Chapter 4 Viruses manipulate vector-host interactions in a host variety specific manner

4.1 Introduction

CMV infection induces different effects on aphids in tobacco and *Arabidopsis thaliana* plants (Ziebell et al., 2011; Westwood et al., 2013a, 2014; Tungadi et al., 2017) (Section 1.10). Before I began this project, a preliminary screen of the effect of CMV infection on aphid-host interaction in various Arabidopsis accessions based on aphid growth rate experiments suggested that there is a continuum of responses to CMV infection with some accessions showing a Type 1 (resistance) response, others showing a Type 2 (susceptibility) response, and some with a neutral response (Groen and Labadie, unpublished data). For example, it was found that CMV infection induces aphid susceptibility on the Arabidopsis accession Ei-2, which contrasts with what was found in Col-0 (Westwood et al., 2013a).

I decided to explore the differences between these two Arabidopsis accessions and effects of CMV infection in detail. I started by comparing colony growth. Subsequently, I tested whether aphid settling (Sections 2.6.7 and 2.6.8), host location (using adhesive trap assays, Section 2.6.6) and feeding behaviour (Section 2.6.9) differ between these accessions. I showed that Arabidopsis accessions have intrinsic differences in aphid attractiveness in the absence of virus infection. I identified a very aphid-attractive Arabidopsis accession, which can potentially be used as a trap plant. Trap plants are plants that are more attractive to aphids and can keep them away from less attractive hosts. In addition, I hypothesise that a trap plant engineered for virus resistance could be used to sanitise viruliferous aphids (See Chapter 5 for full explanation). The results described in this chapter are the

foundation for further experiments described in Chapter 5, which aimed to manipulate and disrupt aphid-mediated transmission of plant viruses.

4.2 Results

4.2.1 Starvation increases frequency of probing by aphids on Arabidopsis

Before starting experiments, I tested whether starvation would increase *M. persicae* probing activities on Arabidopsis Col-0 plants (Section 2.6.4). Previous findings using tobacco showed that starvation of aphids increases the efficiency of aphid transmission of virus to plants (Powell, 1993).

As shown in Figure 4.1, seven-day old starved aphids probed more often (6.22 ± 0.57 times) on Col-0 Arabidopsis leaves than non-starved aphids (1.5 ± 0.46 times) over a five-minute observation period (Student's *t* test, $p < 0.001$). Thus, only starved aphids were used for subsequent aphid behaviour assays such as adhesive trap assays for host location, aphid settling preference and transmission experiments.

4.2.2 The effects of CMV on Arabidopsis-*M. persicae* interactions vary depending on accession

I compared *M. persicae* growth on Col-0 and Ei-2 Arabidopsis plants infected with CMV. One-day-old nymphs were allowed to develop for 5 days on CMV- or mock-inoculated plants of each accession. These nymphs were weighed on the first and on the fifth day of growth to calculate the MRGR (Section 2.6.1). As shown in Figure 4.2A, the MRGR of individual aphids placed on CMV-infected Col-0 plants at 10 dpi (0.36 ± 0.02 mg/mg/day) was significantly lower than that of aphids confined on mock-inoculated

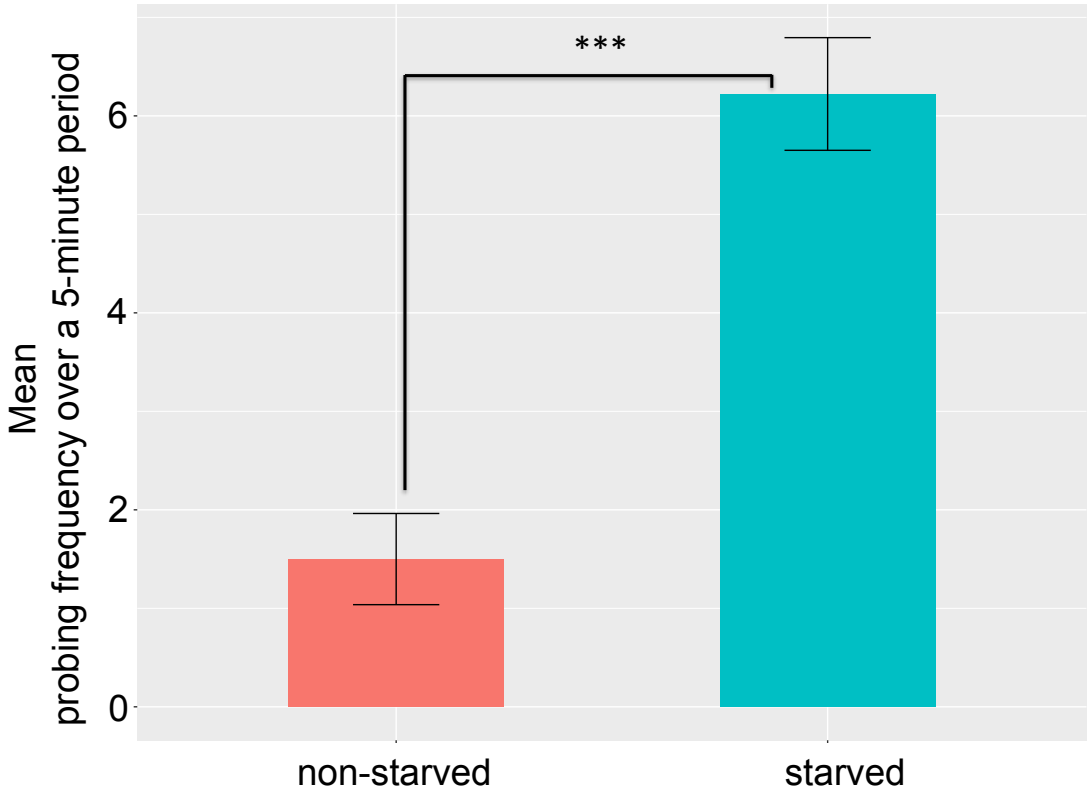


Figure 4.1 Starved aphids probe more frequently than non-starved aphids
Seven-day-old aphids were collected in Petri dishes and starved at 4°C for 10-12 hours. Starved and non-starved aphids were acclimatised for 1 hour before being placed on leaves of healthy Col-0 Arabidopsis plants. Non-starved aphids were directly transferred from aphid stock Chinese cabbage to test plants. Starved aphids displayed a higher number of probing events over a 5-minute period. A total of 10 aphids were observed for each treatment. One individual aphid was used per plant. Error bars denote standard error of the mean. Student's *t* test, *** $p < 0.001$.

plants (0.46 ± 0.02 mg/mg/day) (Student's *t* test, $p<0.001$). However, aphid growth rates on CMV-infected Ei-2 plants (0.36 ± 0.01 mg/mg/day) were similar to those on mock-inoculated (0.39 ± 0.01 mg/mg/day) plants (Student's *t* test, $p=0.082$). Interestingly, aphid growth rates on mock-inoculated Col-0 plants (0.46 ± 0.02 mg/mg/day) were significantly higher than on mock-inoculated Ei-2 plants (0.39 ± 0.01 mg/mg/day). The results observed on CMV-infected Col-0 are consistent with those previously reported (Westwood et al., 2013a). My results found on Ei-2 differed with those observed by Groen and Labadie (unpublished), who found that CMV infection enhanced aphid growth on Ei-2 CMV infected plants (indicating it fitted the profile of Type 2 host). However, I found that CMV infection in Ei-2 had no effect on aphid growth.

I assessed aphid colony growth by recording the number of offsprings produced by individual aphids confined on CMV-infected or mock-inoculated Arabidopsis Col-0 and Ei-2 plants (Section 2.6.2). As shown in Figure 4.2B, at 10 days post infestation, aphid colony growth on CMV-infected Col-0 plants (3.5 ± 0.66) was significantly less than colony growth on mock-inoculated plants (9.3 ± 1.41) (Student's *t* test, $p<0.01$). Colony growth on CMV-infected Ei-2 plants (4.0 ± 0.80) was similar to the aphid colony growth on mock-inoculated plants (6.1 ± 0.75) (Student's *t* test, $p= 0.071$). The results indicate that CMV infection diminishes aphid performance in terms of colony growth on Col-0 but not on Ei-2 plants.

4.2.3 Arabidopsis Ei-2 plants are more attractive to aphids than Col-0 plants

As described in Section 4.2.2, CMV infection induces different responses in host-aphid interactions in two different Arabidopsis accessions. To further study this phenomenon I tested whether aphids settle preferentially on one accession over the other if given the option to choose between Col-0 and Ei-2 plants. For that, I deployed 8 plants equidistantly and alternately distributed in a circle where 25 or 30 aphids were released in the centre of the arena (Section 2.6.8 and Figure 2.7).

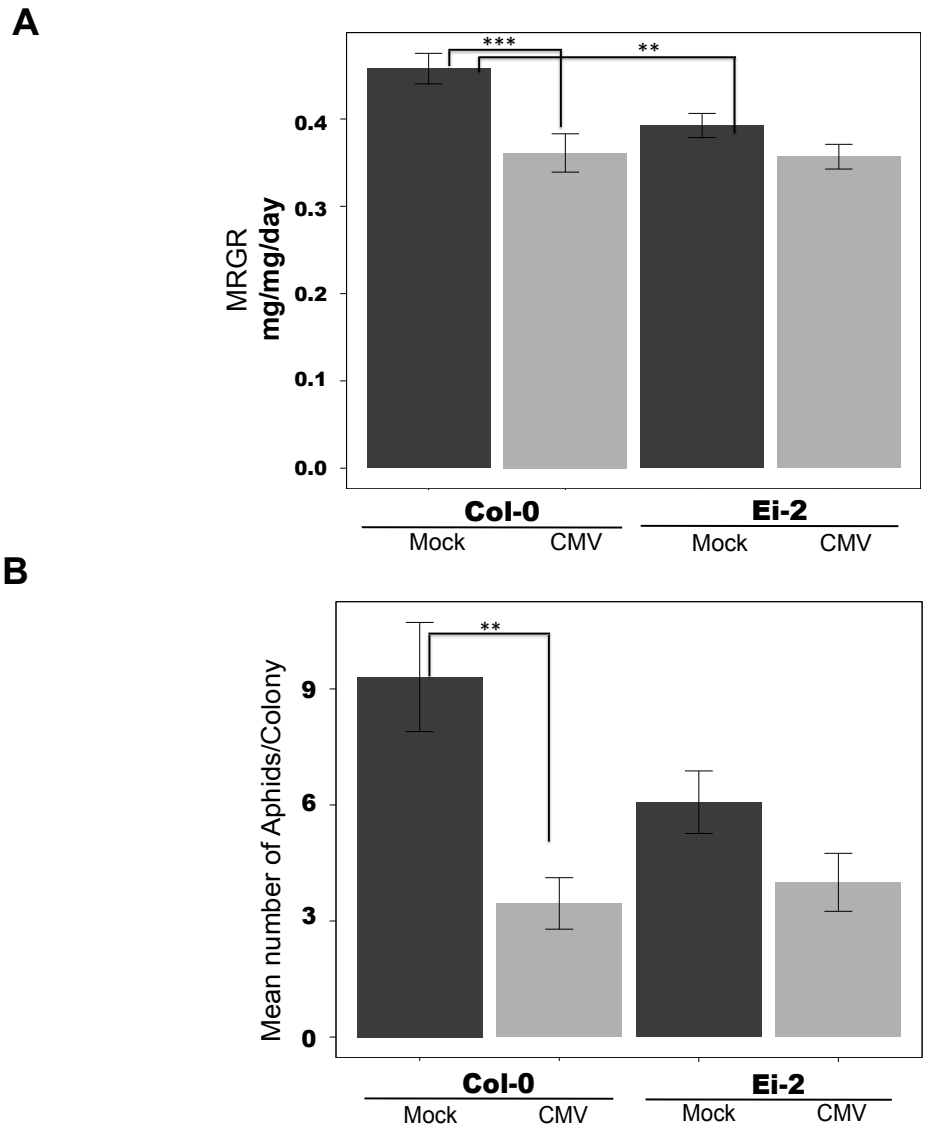


Figure 4.2 The effects of CMV infection on aphid performance varies between Arabidopsis accessions

A shows the result of one independent experiment in which MRGR of aphids was evaluated on CMV-infected and mock-inoculated Col-0 and Ei-2 plants. There was a significant difference between the treatments depending on the accession evaluated. Aphid mean MRGR differed on CMV-infected Col-0 and mock-inoculated Col-0 plants but was similar on CMV-infected and mock-inoculated Ei-2 plants (Student's *t* test, *** $p<0.001$ and $p=0.082$, respectively). Aphid mean MRGR was higher on mock-inoculated Col-0 than mock-inoculated Ei-2. **B** displays the result of one independent experiment in which mean colony size at 10 days post infestation was evaluated on CMV-infected mock-inoculated Col-0 and Ei-2 and plants. Aphid colony size on CMV-infected Col-0 plants was smaller than on mock-inoculated Col-0 plants (Student's *t* test, ** $p<0.01$). Similar aphid colony size was observed on CMV-infected and mock-inoculated Ei-2 plants (Student's *t* test, $p=0.071$). The experiments were performed three times with similar results. (n = 15 aphids).

The total number of aphids found on each plant was recorded after 1 and 24 hours after aphid release. It was assumed that by 24 hours aphids should have settled. As shown in Figure 4.3, aphids settled preferentially on Ei-2 over Col-0 at 1 hour and 24 hour after aphid release.

To further explore the effect of CMV infection on virus-vector-host interactions, I performed experiments in circular pot arenas that combined plants of Arabidopsis accessions Col-0 and Ei-2 that were CMV-infected or mock-inoculated, respectively (Section 2.6.8). As shown in Figure 4.4, at 1 hour following release more aphids (46%) settled on Ei-2 mock-inoculated than (12%) on Col-0 mock-inoculated plants (binomial test, $p < 0.001$). More aphids (29%) settled on Ei-2 CMV-infected plants than (13%) on Col-0 CMV-infected plants (binomial test, $p < 0.001$) (Figure 4.4). Interestingly, in this mixed circular arena aphids had an equal preference for Col-0 CMV-infected (13%) and Col-0 mock-inoculated (12%) plants (Figure 4.4). Aphids (46%) preferred to settle on mock-inoculated Ei-2 than on (29%) CMV-infected Ei-2 plants (binomial test, $p < 0.01$). After 24 hours more aphids (40%) settled on mock-inoculated Ei-2 plants than (31%) on Col-0 mock-inoculated plants (binomial test, $p < 0.05$). In contrast, after 24 hours equal numbers of aphids were found on CMV-infected plants of either accession (15% and 14%) (Figure 4.4). The remarkable conclusion from these experiments is that aphids preferred to settle on CMV-infected Ei-2 to CMV-infected Col-0 at 1 hour after aphid release. The results indicate that there is an initial aphid attraction to Ei-2 plants. The movement of aphids to mock inoculated plants seen after 24 hours was expected, as in previous studies in the lab it was observed that in dual choice test aphids preferred to settle on mock-inoculated Col-0 over CMV-infected plants (Murphy et al., unpublished).

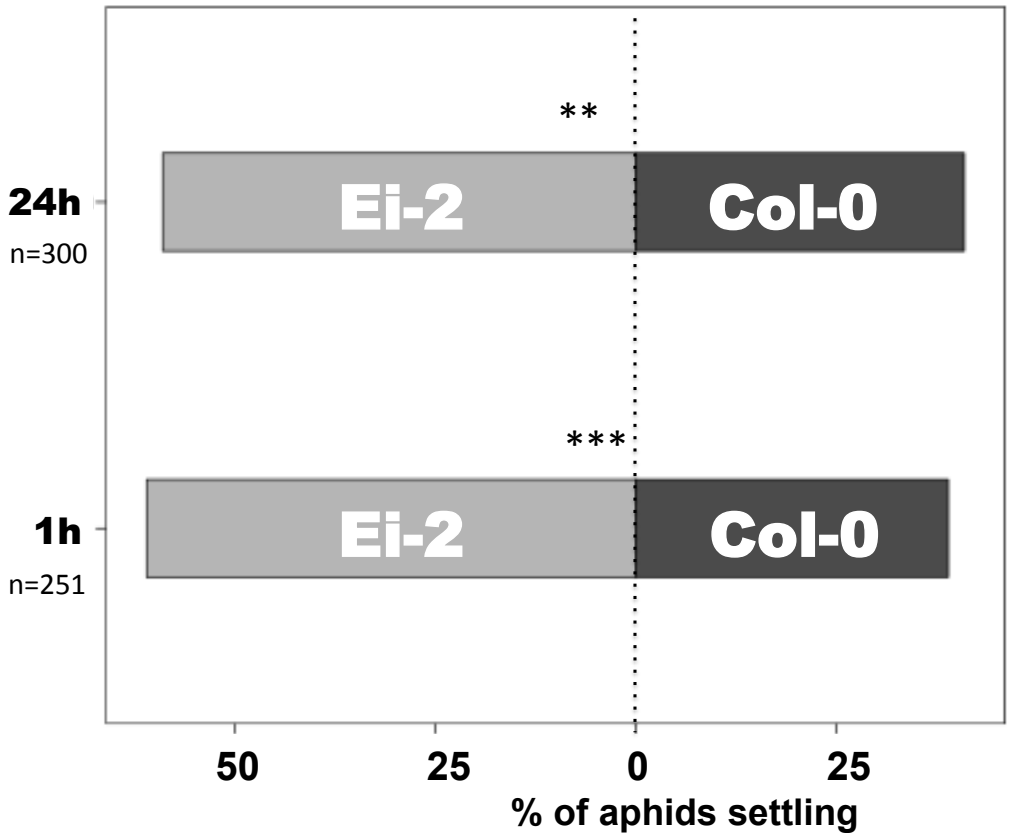
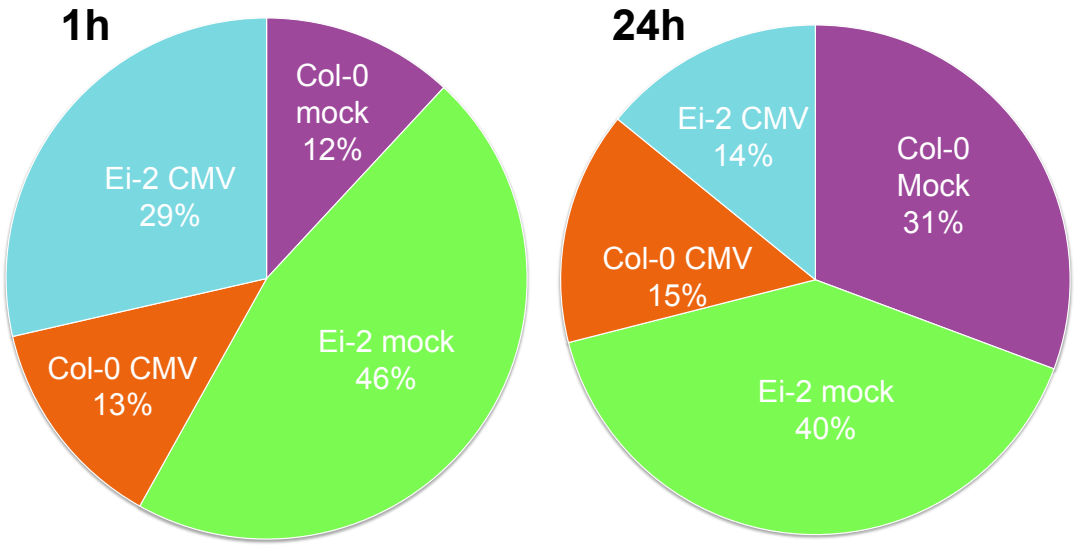


Figure 4.3 More aphids settle on Ei-2 than Col-0 plants

An aphid settling choice test between Col-0 and Ei-2 plants is shown. Aphids (25-30 aphids) were released to make a choice in circular pot arenas containing healthy Col-0 and Ei-2 plants (total 10 pots for bioassay). After 1 hour and 24 hours of release, aphids preferred to settle on Ei-2 plants. Binomial test, ***$p < 0.001$, **$p < 0.01$. The experiment was performed three times with similar results. The total numbers (n) of aphids making a settling choice at each time point were used for analysis.



Pairwise comparisons (% of aphids settling 1h, 24h)		Statistical significance	
		1h (n=210)	24h n=(290)
Col-0 mock 12%, 31%	Ei-2 mock 46%, 40%	***	*
Col-0 CMV 13%, 15%	Ei-2 CMV 29%,14%	***	ns
Col-0 mock 12%, 31%	Col-0 CMV 13%, 15%	ns	***
Ei-2 mock 46%, 40%	Ei-2 CMV 29%, 14%	**	***

Figure 4.4 Aphids prefer Ei-2 to Col-0 regardless of CMV infection
The distribution of aphids found after 1 and 24 hours of release on circular pot arenas containing mock-inoculated and CMV-infected Col-0 plants as well as mock-inoculated and CMV-infected Ei-2 plants is shown (9 pots in total, 25-30 aphids per pot). The numbers of aphids (n) making a settling choice at each time point were used for statistical analysis. At 1 hour after release, aphids settled preferentially on Ei-2 plants regardless of the plants being infected or not. After 24 hours, aphids had settled predominantly on mock-inoculated plants. No statistical significance (ns), *** $p < 0.001$, **$p < 0.01$, *$p < 0.05$, based on binomial test. The experiment was performed three times with similar results.

It was interesting to find that aphids were more attracted to both CMV-infected and mock-inoculated Ei-2 plants than to either Col-0 CMV-infected or mock-inoculated plants (Figure 4.4). The results suggested that Ei-2 was a good candidate to be used as an attractive “decoy” plant to sanitise viruliferous aphids (see Chapter 5).

4.2.4 CMV infection induces specific aphid settling responses to Arabidopsis accessions

I found that Ei-2 plants were intrinsically more attractive to aphids than Col-0 plants (Figures 4.3 and 4.4). These results motivated me to assess whether other Arabidopsis accessions may be even more aphid-attractive than Ei-2 and so potentially better proxies for trap plants (see Chapter 5). Arabidopsis accessions differ in glucosinolate biosynthetic genetic loci and glucosinolate concentrations. This has been proposed as an approach to classify accessions into glucosinolates profiles defined by the concentration and type of glucosinolates (Kliebenstein et al., 2011a) (Table 2.1). Thus, I tested two other accessions, Ler and Cvi, which differ in the genetic loci GS-Elong, and GS-AOP as well as the total glucosinolate concentration as summarised in Table 2.1. Cvi has about two fold more aliphatic glucosinolates than Ei-2 and has a GS-Elong allele that controls the production of three carbon side chains glucosinolates. Cvi has about five fold more aliphatic glucosionales than Col-0 and less than half concentration of indole glucosinolates (Table 2.1).

In contrast, Ler has about half of the concentration of aliphatic glucosinolates than Ei-2 and produces hydroxypropyl glucosinolates rather than alkenyl glucosinolates (Table 2.1). Ler and Col-0 accumulate similar concentrations of aliphatic and indole glucosinolate, but these accessions differ in the GS-Elong and GS-AOP loci. Col-0 has an allele to produce three-carbon side chain glucosinolates whereas Ler has an allele to produce four-carbon side chain glucosinolates (Table 2.1). The phenotypes of the accessions evaluated are shown in Figure 4.5.

I tested whether aphids preferred to settle on Ler and Cvi more than on Col-0 plants (Figure 4.6). Aphids have a similar preference to settle on Cvi or Col-0 and Ler or Col-0 mock-inoculated plants (Figure 4.6A and B). Aphids were found in similar proportions on CMV-infected Cvi and Col-0 or Ler and Col-0 plants. However, more aphids settled on mock-inoculated plants than CMV-infected Cvi, Ler and Col-0 plants after 1 hour and 24 hours of aphid release (Figure 4.6A and B).

In addition, I compared aphid-settling behaviour on Ler and Ei-2, and Cvi and Ei-2 plants (Figure 4.7) to determine whether Ler or Cvi were more attractive to aphids than Ei-2. Aphids had a preference for settling on Cvi over Ei-2 mock-inoculated plants at 1 hour (binomial test, $p < 0.05$), and 24 hours following aphid release (binomial test, $p < 0.001$) (Figure 4.7A). At 1 hour and 24 hours post aphid release, more aphids were found on mock-inoculated plants than on CMV-infected plants of either Ei-2 or Cvi accessions. Similar numbers of aphids of aphids were found on CMV-infected Ei-2 and CMV-infected Cvi plants (Figure 4.7A).

Aphids showed a marked preference to settle on Ei-2 over Ler mock-inoculated plants at 1 hour and 24 hours following aphid release (Figure 4.7B). A similar aphid settling pattern was observed on CMV-infected Ei-2 and Ler plants (Figure 4.7B). More aphids settled on mock-inoculated than on CMV-infected Ler plants at 1 hour following aphid release. However, similar numbers of aphids were found on mock-inoculated and CMV-infected Ler plants 24 hours after aphid release.

Overall, CMV infection of all the Arabidopsis accessions tested induced deterrence to aphid settling. The results also suggest that Ei-2 is a preferred host for aphid settling than the other Arabidopsis accessions evaluated, Col-0 and Ler but not Cvi. In addition, CMV-infected Ei-2 was more attractive to aphids than the others CMV-infected accessions. Thus, Ei-2 was a favourable candidate to be engineered for CMV and TuMV resistance and used as a trap plant to sanitise viruliferous aphids (see Chapter 5).

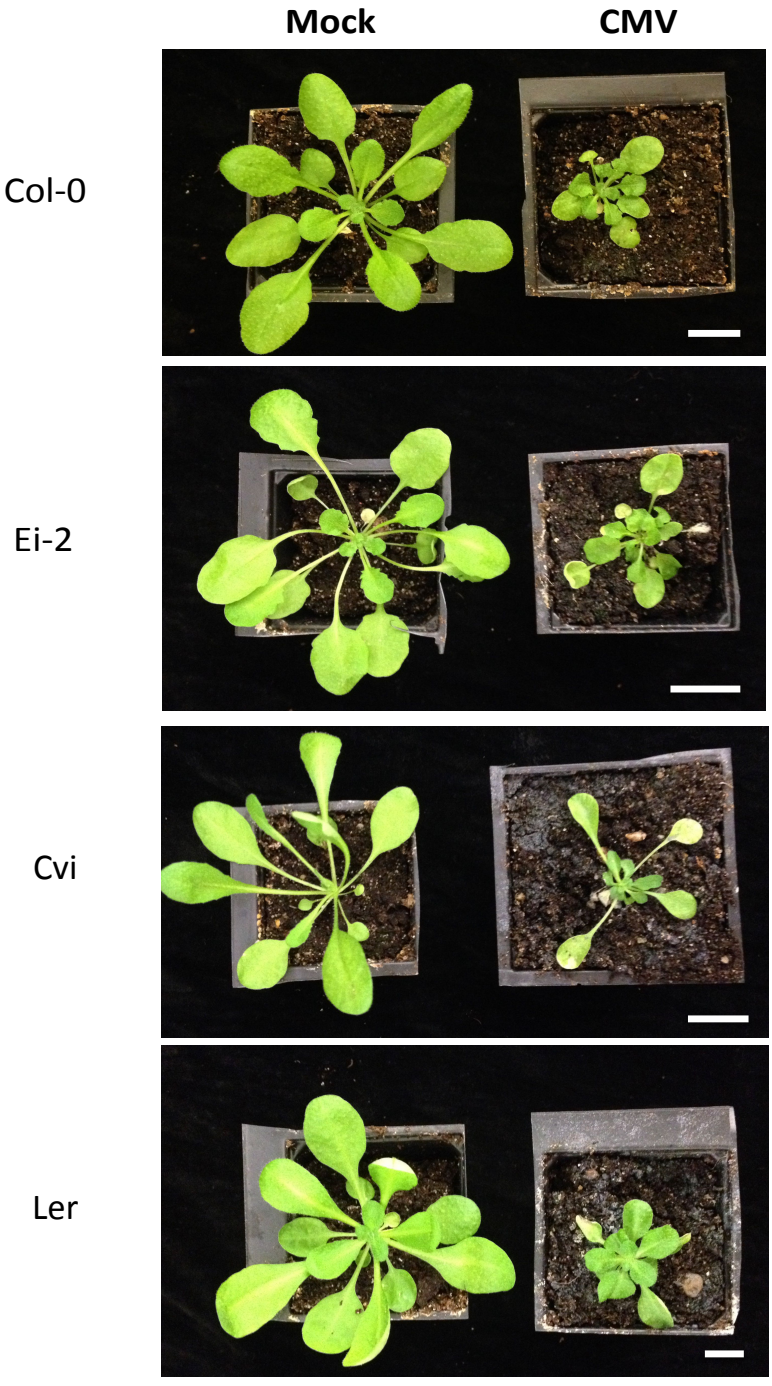
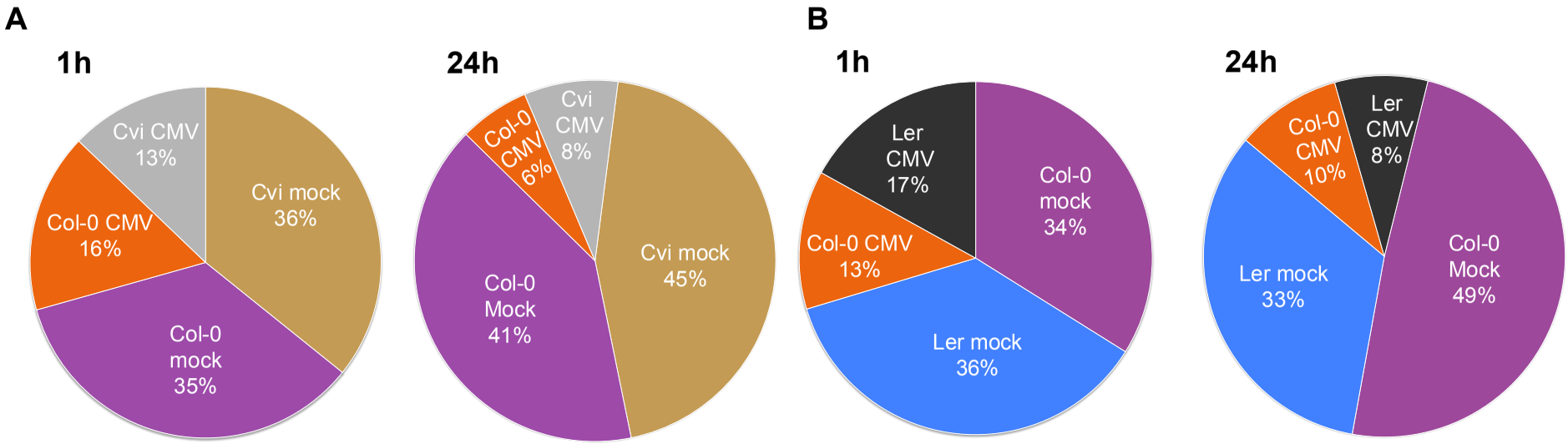


Figure 4.5 Phenotypes and CMV-induced symptoms in four Arabidopsis accessions

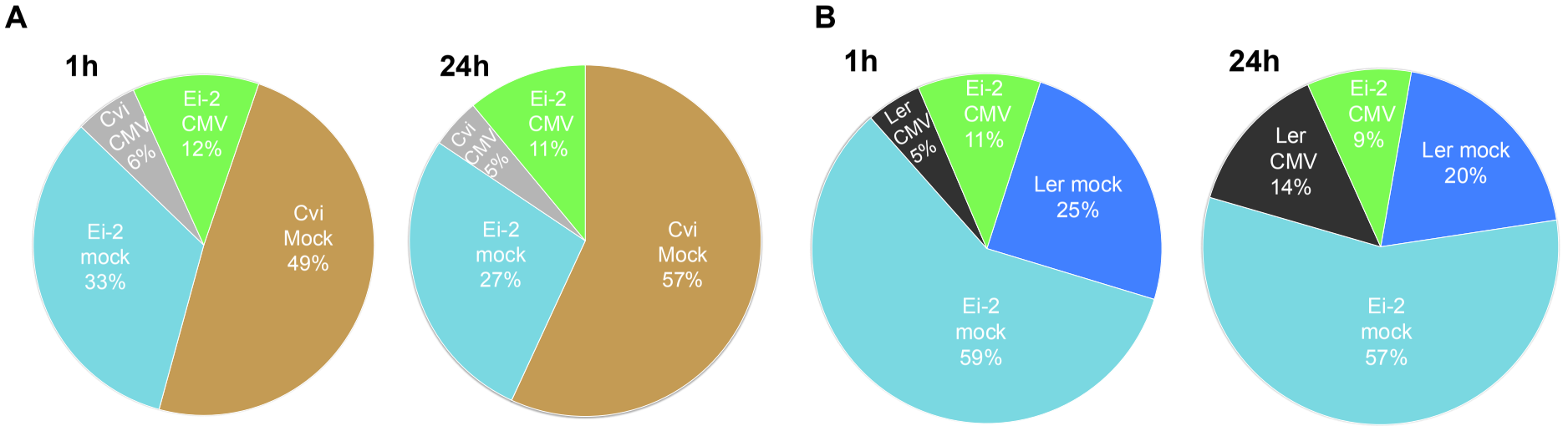
Representative pictures of Col-0, Ei-2, Cvi and Ler plants mock-inoculated (**Mock**) or CMV-infected (**CMV**). Plants were photographed when plants were five weeks old and two weeks post-inoculation. Scale bar, 1 cm.



Pairwise comparisons (% of aphids settling 1h, 24h)		Statistical significance	
		1h (n=109)	24h n=(143)
Col-0 mock 35%, 41%	Cvi mock 36%, 45%	ns	ns
Col-0 CMV 16%, 6%	Cvi CMV 13%, 8%	ns	ns
Col-0 mock 35%, 41%	Col-0 CMV 16%, 6%	*	***
Cvi mock 36%, 45%	Cvi CMV 13%, 8%	*	***

Pairwise comparisons (% of aphids settling 1h, 24h)		Statistical significance	
		1h (n=108)	24h n=(118)
Col-0 mock 34%, 49%	Ler mock 36%, 33%	ns	*
Col-0 CMV 13%, 10%	Ler CMV 17%, 8%	ns	ns
Col-0 mock 34%, 49%	Col-0 CMV 13%, 10%	***	***
Ler mock 36%, 33%	Ler CMV 17%, 8%	**	***

Figure 4.6 Susceptibility to aphid settling of Col-0, Ler and Cvi Arabidopsis plants
Aphids were released in the centre of circular pot arenas (10 pots, 30 aphids per pot). **A.** At 1 hour and 24 hours post release, aphids settled in similar percentages on Cvi or on Col-0 mock-inoculated plants and on CMV-infected Cvi and Col-0 plants. Aphids settled more on mock-inoculated plants than CMV-infected plants of both accession after 1 and 24 hours of aphid release. **B.** Aphids settled similarly on Ler and Col-0 mock-inoculated plants after 1 hour of aphid release, but after 24 hours aphids settled preferentially on Col-0 mock-inoculated than on Ler mock-inoculated plants. After 1 hour and 24 hours of aphid release more aphids settled on mock-inoculated plants than CMV-infected plants of either accession. Similar percentages of aphids settled on CMV-infected plants of both accessions after 1 hour and 24 hours of aphid release. The numbers of aphids (n) making a settling choice at each time point were used for statistical analysis.. Binomial test, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.



Pairwise comparisons (% of aphids settling 1h, 24h)		Statistical significance	
		1h (n=141)	24h n=(154)
Ei-2 mock 33%, 27%	Cvi mock 49%, 57%	*	***
Ei-2 CMV 12%, 11%	Cvi CMV 6%, 5%	ns	ns
Ei-2 mock 33%, 27%	Ei-2 CMV 12%, 11%	***	***
Cvi mock 49%, 57%	Cvi CMV 6%, 5%	***	***

Pairwise comparisons (% of aphids settling 1h, 24h)		Statistical significance	
		1h (n=97)	24h n=(108)
Ei-2 mock 59%, 57%	Ler mock 25%, 20%	***	***
Ei-2 CMV 11%, 9%	Ler CMV 5%, 14%	ns	ns
Ei-2 mock 59%, 57%	Ei-2 CMV 11%, 9%	***	***
Ler mock 25%, 20%	Ler CMV 5%, 14%	**	ns

Figure 4.7 Ei-2 plants are more attractive to aphids than Ler and Cvi plants
Aphids were released in centre of circular pot arenas (10 pots, 30 aphids per pot). **A** After 1 hour of aphid release a higher percentage of aphids were found on Cvi mock-inoculated plants than on Ei-2 mock-inoculated plants. However, after 24 hours of aphid release, aphids settled similarly on both CMV-infected and both mock-inoculated Arabidopsis plants. Aphids settled more on Cvi or Ei-2 mock-inoculated plants than on CMV-infected plants. **B** At 1-hour and 24-hour assessment aphids settled on Ei-2 mock-inoculated plants than on Ler mock-inoculated plants. At 1 hour and 24 hours post aphid release similar percentages of aphids settled on CMV-infected plants of either Ler or Ei-2 accessions. Aphids settled more on Ei-2 mock-inoculated plants than Ei-2 CMV-infected plants after 1 hour and 24 hours of aphid release. At 1 hour-assessment aphids settled preferentially on Ler mock-inoculated plants than on Ler CMV-infected plants, however, similar percentages of aphids were found on mock-inoculated and CMV-infected Ler plants at 24 hours post- aphid release. The number of aphids responding (n) was used for statistical analysis. The numbers of aphids (n) making a settling choice at each time point were used for statistical analysis. Binomial test, *** $p < 0.001$).

4.2.5 CMV infection of Arabidopsis mutant lines compromised in 4MI3M biosynthesis does not encourage aphid settling

CMV infection of Arabidopsis Col-0 plants induces the accumulation of the anti-feedant glucosinolate, 4MI3M and this is associated with decreased growth (MRGR) of aphids placed on CMV-infected plants (Westwood et al., 2013a). Westwood and colleagues (2013) showed that CMV infection of either of two independent *cyp81f2* mutant lines did not induce resistance to aphid growth (MRGR). I hypothesised that the anti-feedant glucosinolate, 4MI3M, might have an effect on aphid settling responses. To further investigate the effect of 4MI3M on the interactions of CMV-infected Arabidopsis plants and *M. persicae*, I conducted aphid settling choice assays.

Aphids were released in the centre of arenas containing wild-type (WT) plants and either of the two *cyp81f2* mutant lines (Figure 4.8A and C); or CMV-infected and mock-inoculated plants of either of the two *cyp81f2* mutant lines (Figure 4.8B and D). Aphids had equal preference for WT or *cyp81f2-1* plants (Figure 4.8A). However, aphids settled more on the *cyp81f2-2* than WT plants (Figure 4.8C). Interestingly, aphids settled more on mock-inoculated plants than CMV-infected plants of either *cyp81f2* mutant lines (Figure 4.8B and D), which is similar to the aphid settling observed in mock-inoculated and CMV-infected WT plants (Figure 4.8E). The results suggest that 4MI3M cannot entirely explain aphid settlement preference for mock-inoculated plants than CMV-infected plants.

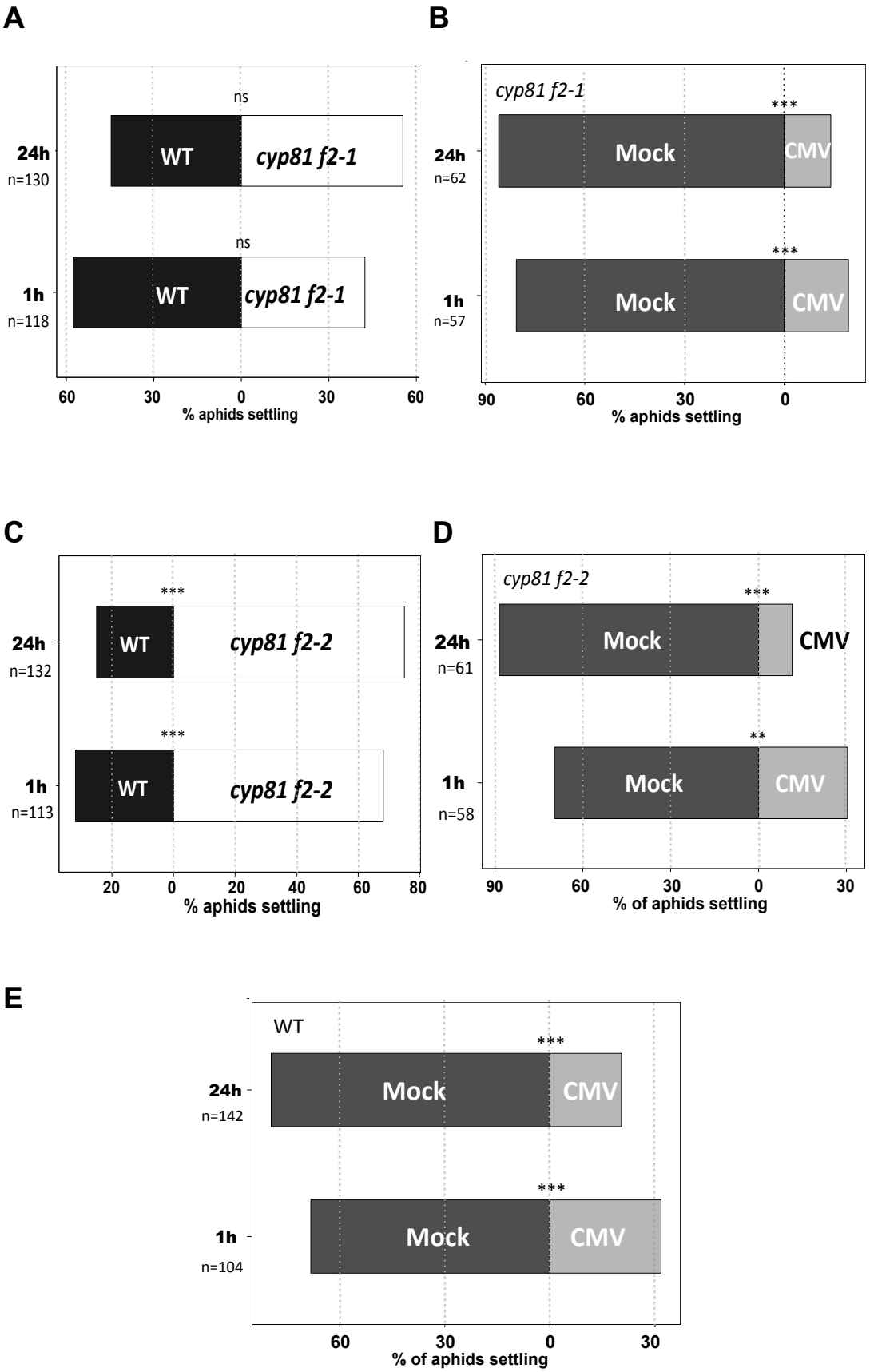

A
ns
24h
n=130
WT
cyp81 f2-1
ns
1h
n=118
WT
cyp81 f2-1
60
30
0
30
60
% aphids settling
B
cyp81 f2-1
***
24h
n=62
Mock
CMV
***
1h
n=57
Mock
CMV
90
60
30
0
% aphids settling
C
***
24h
n=132
WT
cyp81 f2-2
***
1h
n=113
WT
cyp81 f2-2
20
0
20
40
60
80
% aphids settling
D
cyp81 f2-2
***
24h
n=61
Mock
CMV
**
1h
n=58
Mock
CMV
90
60
30
0
30
% of aphids settling
E
WT
***
24h
n=142
Mock
CMV
***
1h
n=104
Mock
CMV
60
30
0
30
% of aphids settling

Figure 4.8 The feeding deterrent glucosinolate 4MI3M is not the sole determinant of deterrence to aphid settling in free choice assays
Thirty aphids were released in the centres of the two-way arenas (10 pots per experiment, 25-30 aphids per pot, Figure 2.7A) containing WT (wild-type) plants and mutant plants unable to synthesise normal levels of 4MI3M (either *cyp81 f2-1* or *cyp81 f2-2)* (**A** and **C**), arenas containing CMV-infected and mock-inoculated plants of either of the two *cyp81 f2* mutant lines (**B** and **D**). **A** Aphids settled equally on untouched *cyp81 f2-1* and WT plants at 1 hour and 24 hours after release. **B** Aphids preferred to settle on mock-inoculated *cyp81 f2-1* than CMV-infected *cyp81 f2-1* plants at 1 hour and 24 hours after release. **C** More aphids settled on *cyp81 f2-2* than on WT plants at both time points after release. **D** Aphids preferred to settle on mock-inoculated *cyp81 f2-2* than mock-inoculated plants. **E** More aphids settled on mock-inoculated than on CMV-infected WT plants (control). The numbers of aphids (n) making a settling choice at each time point were used for statistical analysis. Binomial test, *** $p < 0.001$ and ** $p < 0.01$.

4.2.6 Aphids are initially attracted to CMV-infected Arabidopsis plants

Aphids are inhibited from feeding on CMV-infected Arabidopsis Col-0 based on EPG experiments (Westwood et al., 2013a). I found that in aphid free-choice settling experiments described in Sections 4.2.3-4.2.5 aphids preferred to settle on mock-inoculated Arabidopsis plants. Even though aphid performance and behaviour evaluated by Westwood and colleagues (2013) and the results described in Sections 4.2.3-4.25 show that CMV infection of Arabidopsis plants induces aphid deterrence in some accessions such as Col-0 or no effect in Ei-2, it was still unclear to me how CMV encourages its transmission by *M. persicae*.

That question led me to test whether host location by *M persicae* was different between CMV-infected and mock-inoculated plants of different Arabidopsis accessions. For that, I performed adhesive trap assays under dark and light conditions (Section 2.6.6 and Figure 2.6). The assay set-up limited the assessment of aphid attraction to odours (dark conditions) and visual and/or odour cues (light conditions) emitted by the host. Thus, aphids responding to odours and/or visual cues were trapped on the adhesive tape. Under dark conditions, I conducted time course assays with observations at intervals of 10 minutes over a one-hour period (Figure 4.9). There was a trend showing that more aphids were moving towards CMV-infected plants than to mock-inoculated plants in both Arabidopsis accessions Col-0 and Ei-2 plants (Figure 4.9A and B). Interestingly, when the assay was performed under light conditions (Figure 4.10), I found that aphids had a preference for CMV-infected plants of both Arabidopsis accessions. At very early time points, 10 minutes, similar proportions of aphids preferred CMV-infected and mock-inoculated plants. However, the preference for CMV-infected plants of both accessions was stronger at 20 minutes and 30 minutes post-aphid release as more responding aphids were trapped on the adhesive tape.

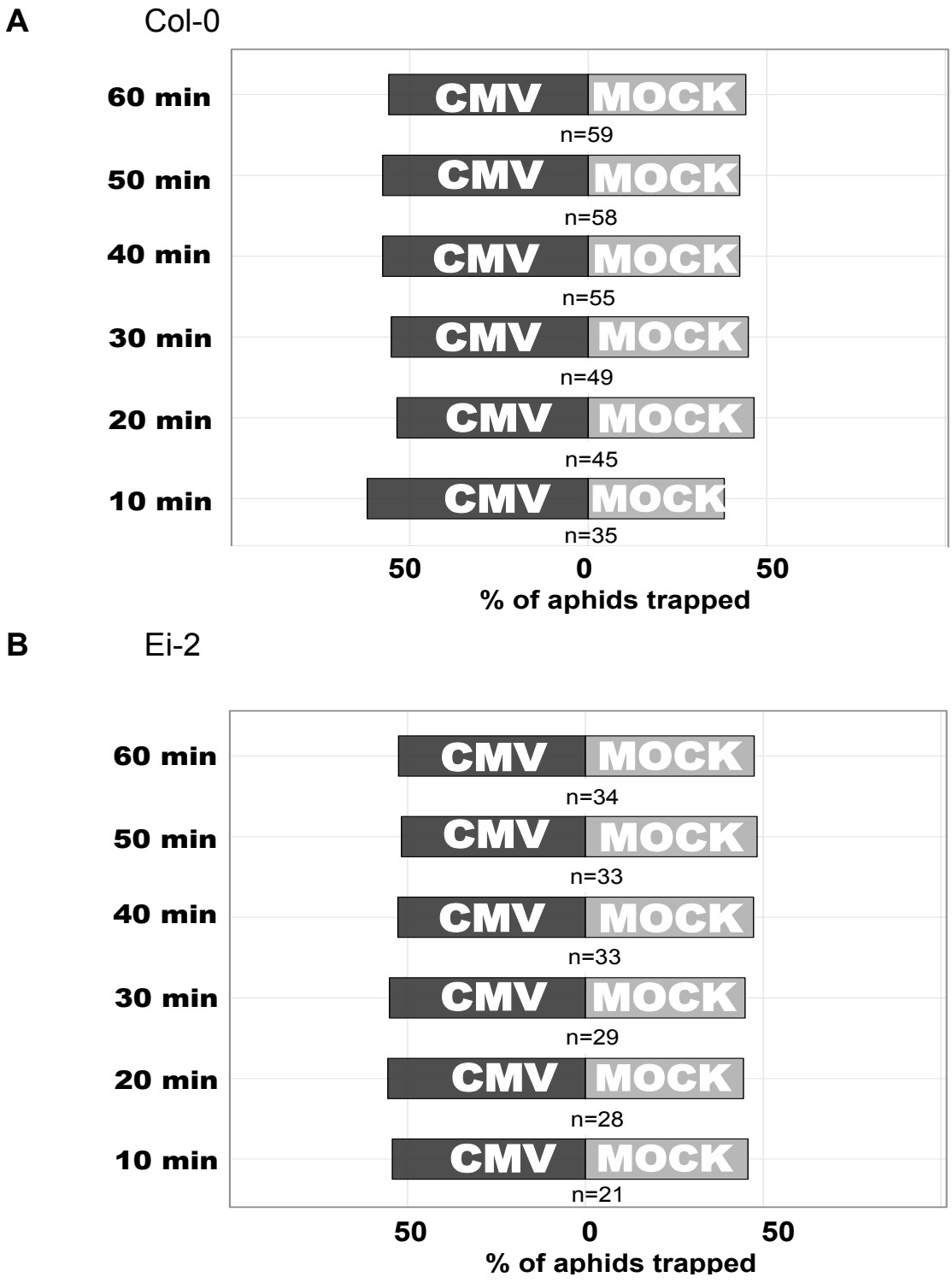


Figure 4.9 Adhesive trapping experiment: host location under dark conditions
Aphids were released in the centre of arenas each containing a CMV-infected and a mock-inoculated plant (10 pots, 25 aphids per pot). An adhesive tape was placed between the test plants (Figure 2.6). These experiments were performed under **dark** conditions. **A** CMV-infected Col-0 plants and **B** CMV-infected Ei-2 plants showed a trend to attract more aphids than mock-inoculated plants. Binomial test showed no statistical significance. The results displayed are the cumulative number of aphids (n) responding at each time point. The total number of responding aphids was pooled and used for statistical analysis.

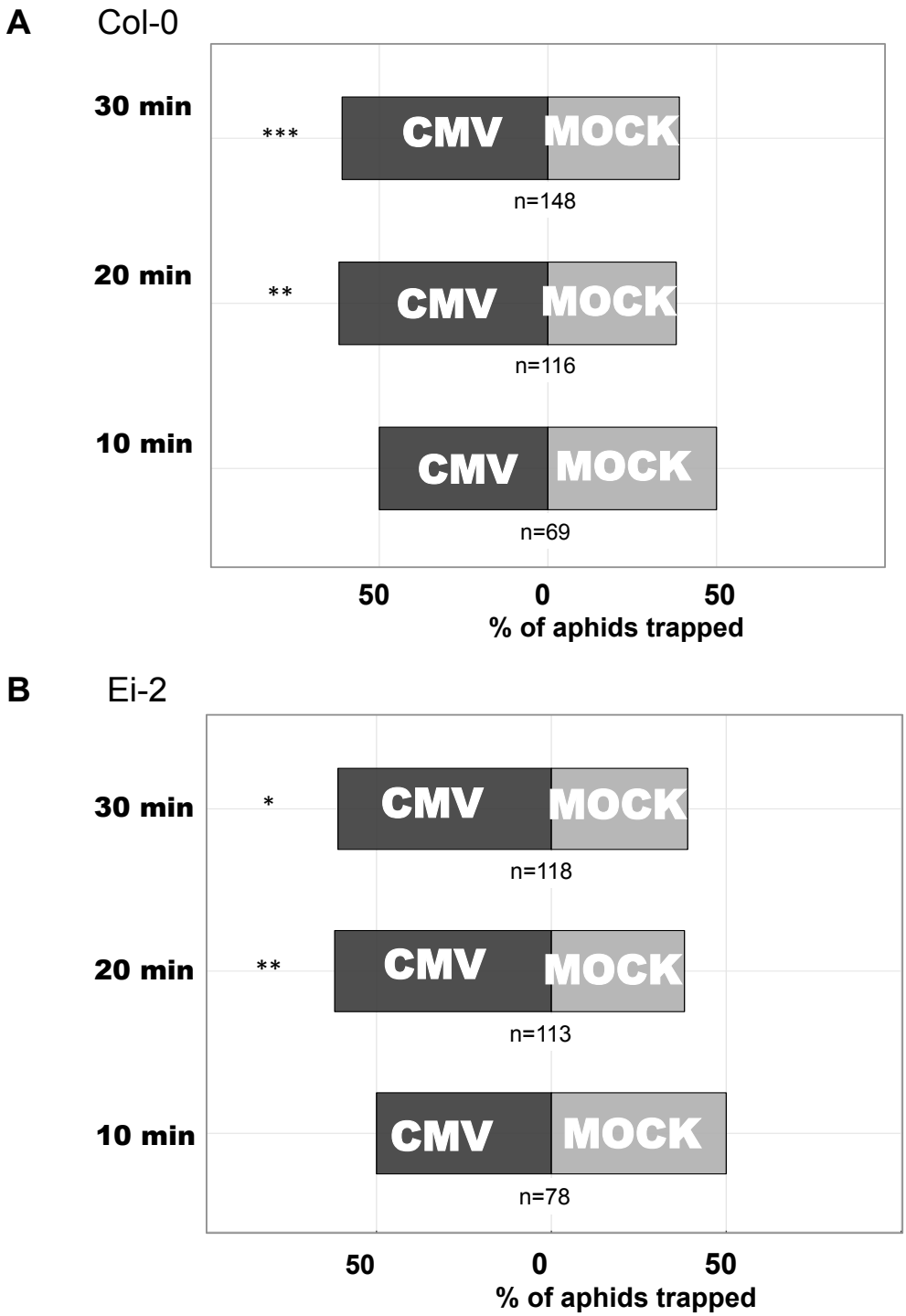


Figure 4.10 Adhesive trapping experiment: aphids are attracted to the odours or visual cues emitted by CMV- infected plants

Aphids were released in the centre of arenas containing each a CMV-infected and a mock-inoculated plant (10 pots, 25 aphids per pot). An adhesive tape was placed between the test plants (Figure 2.6). These experiments were performed under **light** conditions observations were made at 10, 20 and 30 minutes after release. **A** Aphids showed preference for cues emitted by Col-0 CMV-infected plants after 20 min of aphid release. **B** At 10 minutes post aphid release aphids preferred equally Ei-2 CMV-infected and Ei-2 mock- inoculated plants. By 20 and 30 minutes post aphid release, more aphids had moved towards Ei-2 CMV-infected plants. The total number of responding aphids was pooled and used for statistical analysis. Binomial test, *** $p < 0.001$, ** $p < 0.01$ and *$p < 0.05$.

To further complement these experiments, I also performed a Y-tube olfactometry assay (Section 2.6.5, Figure 2.5). I tested the attraction of aphids to the odours emitted by CMV-infected and TuMV-infected plants. As noted in Figure 4.11, aphids showed a strong preference for odour cues from CMV-infected and TuMV-infected plants (binomial test, $p < 0.001$). The results show that volatile cues emitted by CMV-infected and TuMV-infected plants are more attractive to aphids than volatile cues emitted by mock-inoculated plants.

4.2.7 CMV infection induces qualitative and quantitative changes in the VOC blend emitted by Arabidopsis plants

As described in previous sections, aphids were more attracted to volatile cues emitted by CMV-infected plants. To investigate the potential role of chemical cues emitted by CMV-infected Col-0 and Ei-2 plants, I conducted experiments to analyse VOC profiles of Col-0 and Ei-2 plants. For that, Arabidopsis plant headspace volatiles were collected and analysed by GC-MS (Section 2.8).

Principal component analysis was used to compare the mass spectra of the VOCs emitted by CMV-infected and mock-inoculated Col-0 and Ei-2 plants (Section 2.8). PCA showed that the emitted VOCs were distinct between both accessions. PC1 discriminated the differences between Col-0 and Ei-2 mock-inoculated plants and PC2 discriminated between mock-inoculated and CMV-infected plants of both accessions (Figure 4.12). The results indicate that there are qualitative differences between the volatile blends emitted by Col-0 and Ei-2 plants and those blends also differ when the plants are CMV-infected. This could explain the ability of aphids to discriminate between Col-0 and Ei-2 plants either mock-inoculated or CMV-infected as shown in aphid settling and adhesive trapping assays (Section 4.2.4 and 4.26).

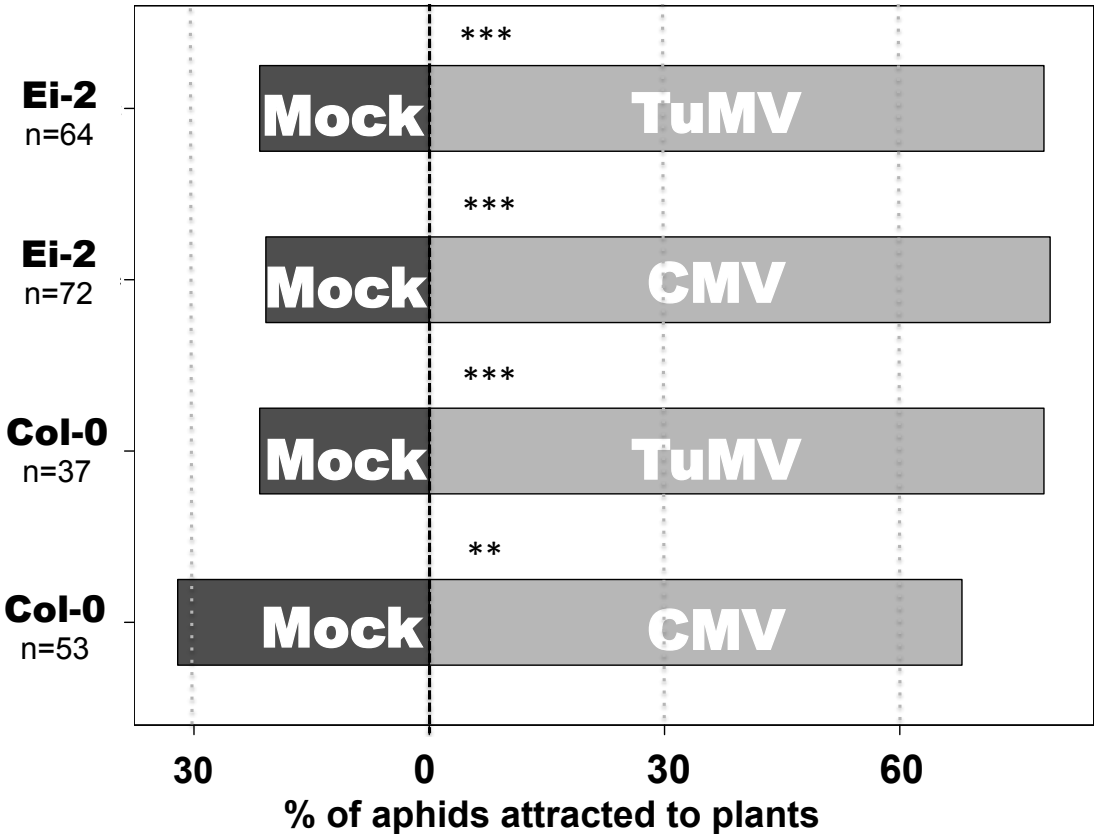


Figure 4.11 Y-tube Olfactometry: CMV-infected and TuMV-infected Arabidopsis plants emit plant volatiles that attract aphids
Responses of *M. persicae* to volatiles emitted from CMV-infected or TuMV-infected Arabidopsis Col-0 and Ei-2 plants were evaluated in Y-tube olfactometry assays. Each assay consisted of a virus-infected (CMV or TuMV) Arabidopsis plant and mock-inoculated plant (Col-0 or Ei-2). Twenty-five 7-day old aphids were released at the end of the main arm of the Y-tube and allowed to crawl freely for 1 hour. Aphids that chose to walk into one of the Y-tube arms were considered to have made a choice (n), and only these were used in statistical analysis. A total of 5 dual-choice assays were performed per combination and 25 aphids per combination. Statistical differences based on binomial test, *** $p < 0.001$ and ** $p < 0.01$.

I compared the chromatograms of each sample group in order to tentatively identify and quantify the VOCs emitted by mock-inoculated and CMV-infected Col-0 and Ei-2 plants. Chromatograms of mock-inoculated and CMV-infected Col-0 and Ei-2 plants showed similar VOC profiles (Figure 4.13). Four peaks were provisionally identified as compounds known to be involved in plant-insect interactions by comparison of their mass spectra with the database available within the Xcalibur software (Section 2.8)

The four peaks were identified as 3-methyl-hexane, (*E*)-2-hexene-1-ol, 2-octanone and 3-pentanol (Figure 4.13). The peak area units of each provisionally identified compound were used to estimate its relative abundance in emissions from CMV-infected and mock-inoculated plants of both accessions (Figure 4.14). I found that 3-methyl-hexane emission was lower by half in CMV-infected Col-0 plants compared to mock-inoculated Col-0 plants, whereas CMV-infected Ei-2 plants emitted twice as much 3-methyl-hexane as mock-inoculated Ei-2 plants (Figure 4.14A).

Similarly, 2-octanone emissions appeared to be particularly elevated in CMV-infected Ei-2 plants (Figure 4.14C). Thus differences in 3-methyl-hexane and 2-octanone emissions could contribute to the attractiveness of CMV-infected Ei-2 plants. The other two identified compounds, (*E*)-2-hexene-1-ol and 3-pentanol, were not significantly different between accessions or CMV-infected and mock-inoculated plants (Figure 4.14B and D). In these experiments, the relative abundance of VOCs in the headspace around Arabidopsis plants was estimated. The abundance of VOCs was not normalised to the fresh or dry mass of the plants and therefore reflects the differences that an aphid can perceive. Mock-inoculated Ei-2 plants appear to be bigger than Col-0 plants (Figure 4.5) reflected in their increased dry mass (Figure 4.15).

CMV infection of both Col-0 and Ei-2 appears to have a significant effect on the size of the plants, but does not greatly reduce the dry weight (Figure 4.15). This indicates that differences in the abundance of certain VOCs emitted by virus or mock-inoculated plants cannot simply be attributed to the size of the plants. Overall, the results suggest that Col-0 and Ei-2 plants, either CMV-infected or mock- inoculated, emit distinct VOC blends. Although the small number of tentatively identified compounds may not fully explain the different blends, the behavioural assays showed that aphids can perceive the differences between these two accessions. Further studies are needed to confirm the identity of these compounds and other unidentified compounds that might elucidate which volatiles are causing changes in aphid behaviour.

4.2.8 CMV infection induces changes in aphid feeding behaviour in an accession-specific manner

Aphids showed less sustained phloem feeding on Col-0 plants infected with CMV (Westwood et. al, 2013a). I performed EPG experiments to compare aphid feeding behaviour on Col-0 and Ei-2 plants (Section 2.6.9). The following waveform patterns were selected from the EPG recordings for analysis: time to first probe (i.e time from the beginning of the recording to the start of the first probe); duration of the first probe; time to first E1/E2 from the beginning of the EPG; total duration of phloem feeding (E); total duration of phloem salivation (E1) and total duration of phloem ingestion (E2) (Table 2.3).

Time to the first probe was significantly briefer on CMV-infected Col-0 (38.30 ± 8.17 minutes) plants than on mock-inoculated Col-0 plants (156.91 ± 34.64 minutes) (LSD test, α=0.05) (Figure 4.16A). In contrast, the time to first probe was similar for aphids feeding from CMV-infected and mock-inoculated Ei-2 plants (Figure 4.16A).

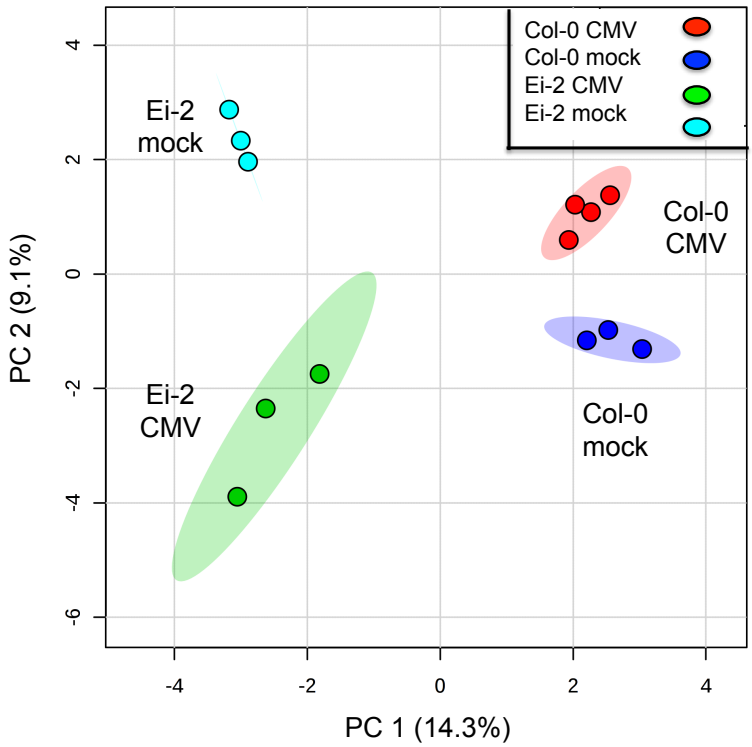


Figure 4.12 Changes in VOC emission by Col-0 and Ei-2 Arabidopsis plants after CMV infection

Principal component analysis of m/z values (over 75 Da) obtained by GC-MS of VOCs samples from Arabidopsis plants. The samples were collected by dynamic headspace trapping from Arabidopsis plants as follows: mock-inoculated Col-0 (blue), CMV-infected Col-0 (red), mock-inoculated Ei-2 (green) and CMV-infected Ei-2 (cyan). The principal component analysis separated each accession and treatment into four different clusters. The plot shows the percentage of variation of the data in two principal components, 14.3% for PC1 and 9.1% for PC 2.

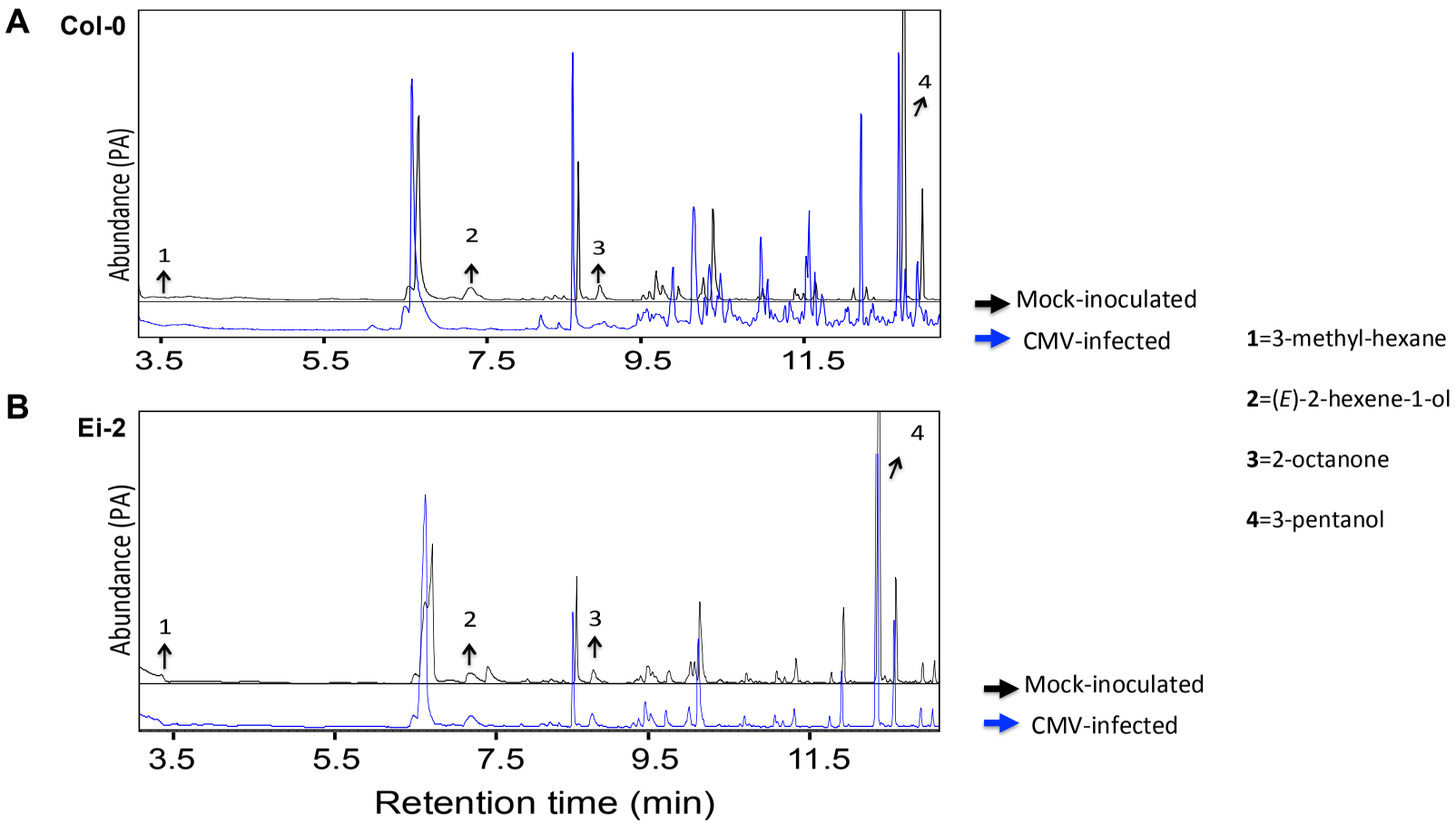
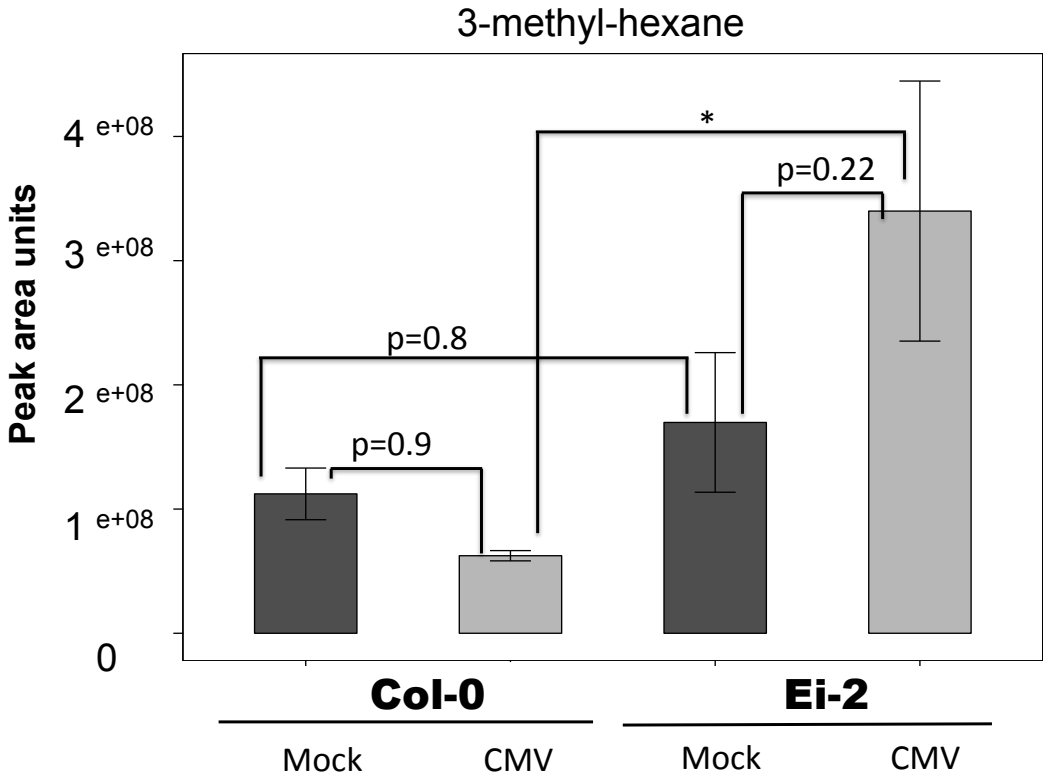


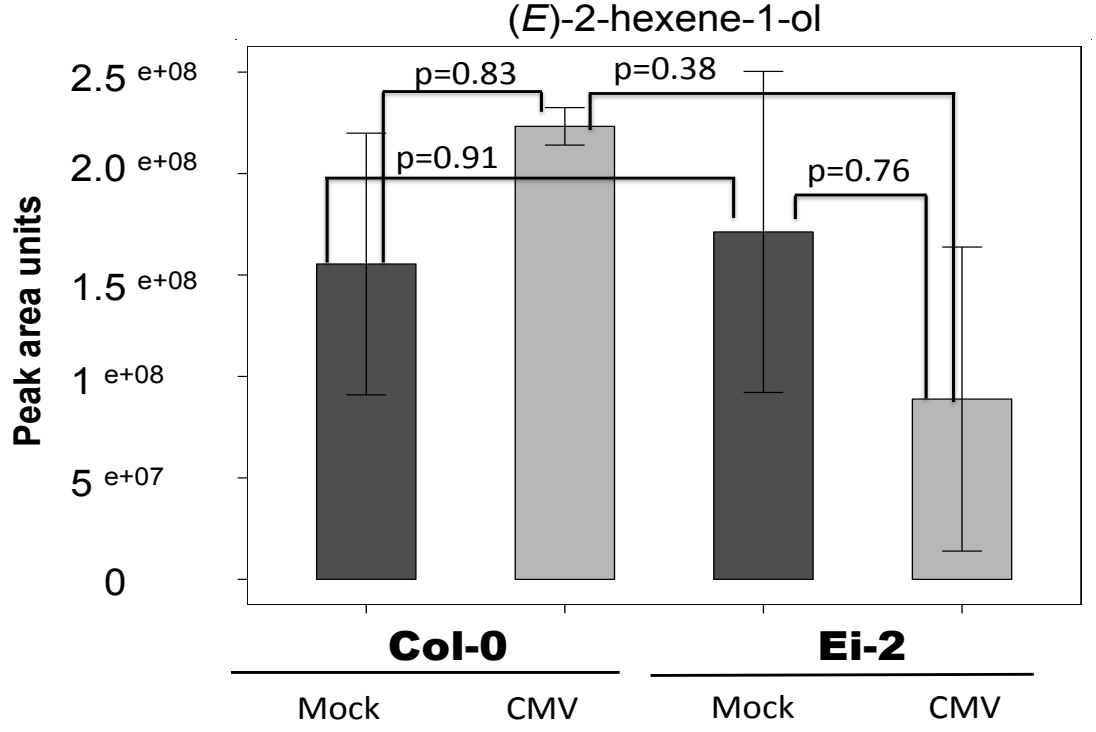
Figure 4.13 Representative gas chromatograms of VOCs emitted by Arabidopsis plants

Eluted peaks and retention times are shown in the gas chromatograms. Four compounds were identified by comparing their mass spectra with mass spectra available in the Xcalibur Library Browser as follows: **1**=3-methyl-hexane, **2**=(*E*)-2-hexene-1-ol, **3**=2-octanone and **4**=3-pentanol. **A** VOC chromatograms from mock-inoculated and CMV-infected Col-0 plants **B.** VOC chromatograms from mock-inoculated and CMV-infected Ei-2 plants.

A


3-methyl-hexane
Peak area units
4 e+08
3 e+08
2 e+08
1 e+08
0
*
p=0.22
p=0.8
p=0.9
Col-0
Ei-2
Mock
CMV
Mock
CMV

B


(E)-2-hexene-1-ol
Peak area units
2.5 e+08
2.0 e+08
1.5 e+08
1 e+08
5 e+07
0
p=0.83
p=0.38
p=0.91
p=0.76
Col-0
Ei-2
Mock
CMV
Mock
CMV

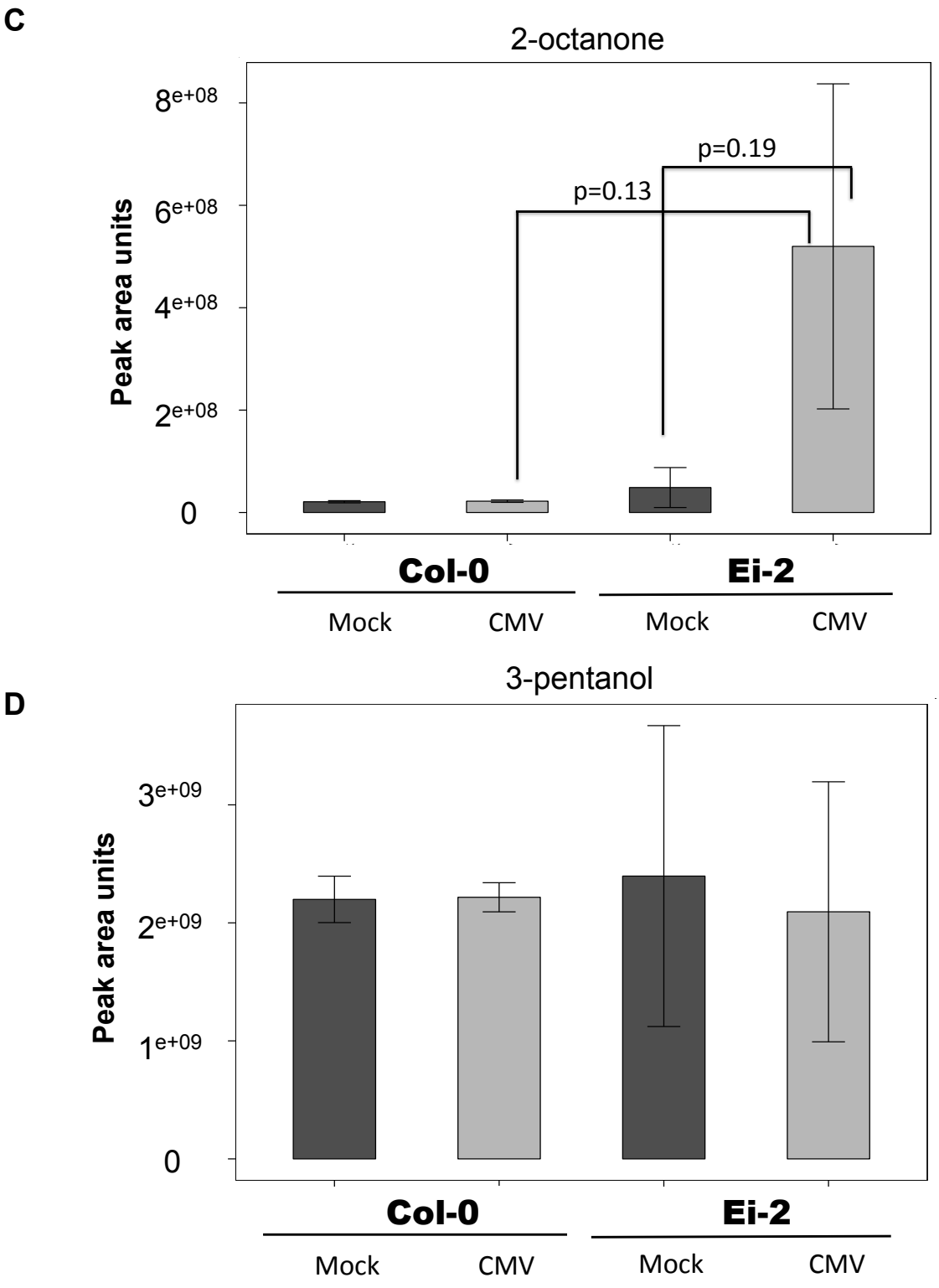


Figure 4.14 Relative emission of individual VOCs from Arabidopsis plants over 24 hours entraiment

The mean peak areas units are shown of the identified volatiles **A** 3-methyl-hexane, **B** (*E*)-2-hexene-1-ol, **C** 2-octanone and **D** 3-pentanol of mock-inoculated and CMV-infected of both Col-0 and Ei-2 plants. Error bars represent standard error of the mean (n = 3 pots containing 19 plants). The level of significance is shown by the p-value calculated with one-way ANOVA and post-hoc Tukey test.

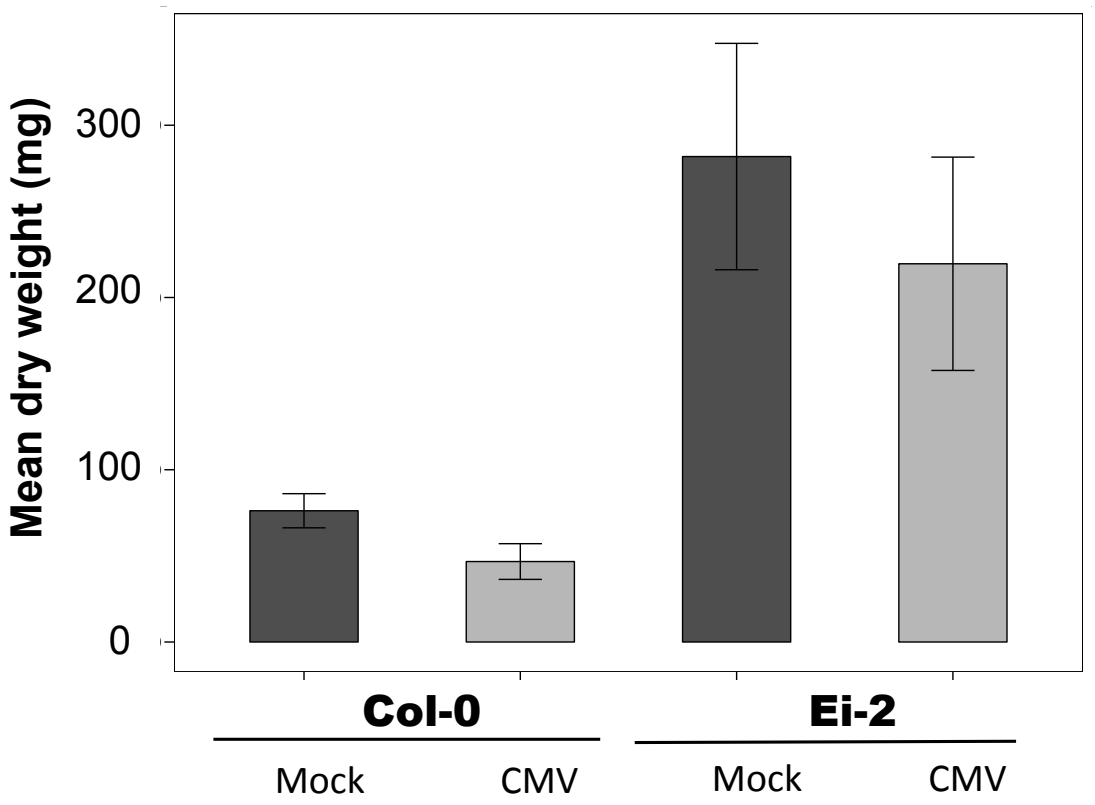


Figure 4.15 CMV infection does not greatly affect the dry weight of Arabidopsis plants

The dry weight of the Col-0 and Ei-2 plants used for VOCs assays is shown. The mass of tissue is expressed in milligrams of dry weight. Error bars denote standard error of the mean (n = 3, each sample comprised 19 Arabidopsis plants). No significant differences found by One-Way ANOVA test

Interestingly, the time to first probe from start of EPG was shorter on mock-inoculated Ei-2 plants (63.31 ± 11.5 minutes) than on mock-inoculated Col-0 plants (156.91 ± 34.64 minutes) (LSD test, α=0.05) (Figure 4.16A).

Aphids showed a very short duration of the first probe on CMV-infected Col-0 plants (27.05 ± 3.57 minutes) whereas the duration of the first probe was longer on mock-inoculated Col-0 plants (237.46 ± 57.89 minutes) (LSD test, α=0.05) (Figure 4.16B). The duration of the first probe on CMV-infected Ei-2 plants (24.46 ± 4.43 minutes) was similar to mock-inoculated Ei-2 plants (27.29 ± 4.43 minutes) (Figure 4.16B). Interestingly, the time to first probe (156.91 ± 34.64 minutes) on mock-inoculated Col-0 plants was longer compared with the time to first probe (63.31 ± 11.5 minutes) on mock-inoculated Ei-2 plants (Figure 4.16A) (LSD test, α=0.05). Similarly, the duration of the first probe (237.46 ± 57.89 minutes) was longer on mock-inoculated Col-0 plants than on mock-inoculated Ei-2 plants (27.29 ± 4.43 minutes) (Figure 4.16A and B) (LSD test, α=0.05).

Thus, the results on time to first probe and duration of first probe on aphid probing behaviour suggest that there are intrinsic differences between Col-0 and Ei-2, which indicate that there might be resistance factors located in the surface of the plant. As shown in Figure 4.17A-D, aphids had difficulties reaching phloem of CMV-infected Col-0 plants. Time to first E1/E2 was significantly higher on CMV-infected Col-0 plants (163.8 ± 22.37 minutes) than on mock-inoculated Col-0 plants (97.85 ± 20.75 minutes) (LSD test, α=0.05) (Figure 4.17A). Although the time to first E1/E2 was higher on CMV-infected Ei-2 plants (129.91 ± 22.17 minutes) than on mock-inoculated Ei-2 plants (88.6 ± 28.05 minutes), it was not statistically significant (LSD test, α=0.05) (Figure 4.17A). Aphids experienced a significantly reduced total duration of phloem feeding on CMV-infected Col-0 plants (52.19 ± 10.01 minutes),

mock-inoculated Ei-2 plants (56.34 ± 18.38 minutes) and CMV-infected Ei-2 plants (52.44 ± 18.85 minutes) compared to mock-inoculated Col-0 plants (114.75 ± 24.88 minutes) (LSD test, α=0.05) (Figure 4.17B). Following the same trend, the total duration of phloem salivation was higher on mock-inoculated Col-plants (7.25 ± 2.28 minutes) than either CMV-infected Col-0 plants (5.18 ± 0.82 minutes) or mock-inoculated (5.87 ± 1.16 minutes) or CMV-infected (7.02 ± 2.22 minutes) Ei-2 plants. Surprisingly, the total duration of phloem ingestion also followed the same trend but again the differences were not statistically significant. The total duration of phloem ingestion was higher on mock-inoculated Col-plants (122.01 ± 27.71 minutes) than either CMV-infected Col-0 plants (110.57 ± 27.73 minutes) or mock-inoculated (75.71 ± 23.42 minutes) or CMV-infected (61.93 ± 24 minutes) Ei-2 plants.

It is important to point out that the percentage of aphids exhibiting the total duration of phloem ingestion was lower in all the samples as follows: 82% on mock-inoculated Col-0 plants, 75% on CMV-infected Col-0 plants, 63% on mock-inoculated Ei-2 plants and 71% on CMV-infected Ei-2 plants.

I consistently found that aphids experienced difficulties to maintain a sustained feeding on CMV-infected Col-0 plants. Unexpectedly, the assessment of aphid feeding behaviour showed that aphids encounter difficulties to reach the phloem on Ei-2 plants and that behaviour was similar in CMV-infected and mock-inoculated Ei-2 plants. Taken together, the results suggest that CMV-infection changes aphid feeding behaviour at phloem level in an accession specific manner in Arabidopsis plants.

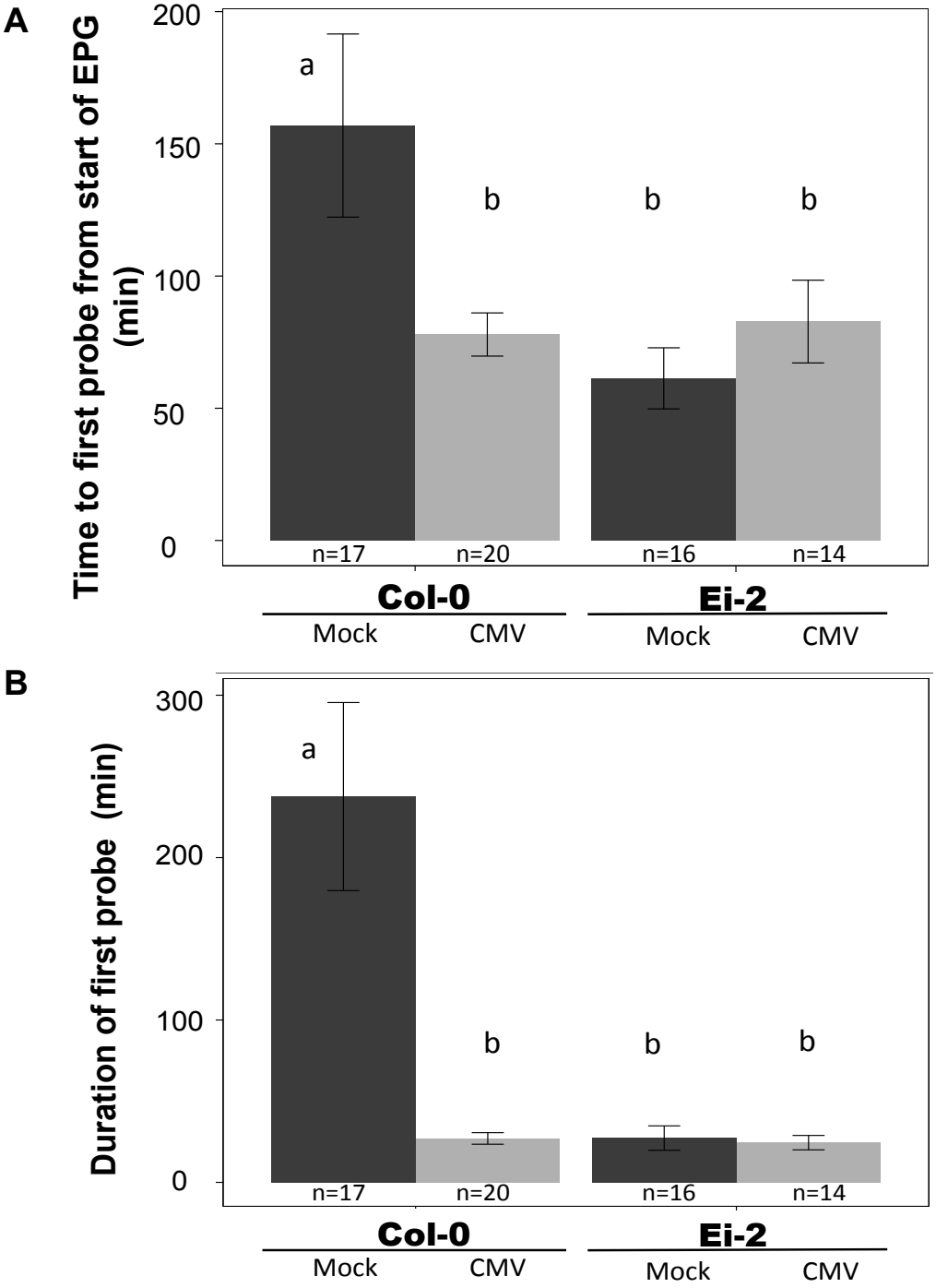


Figure 4.16 EPG: CMV infection stimulates probing in Col-0 plants
M. persicae feeding behaviour was evaluated on CMV-infected and mock-inoculated Col-0 and Ei-2 Arabidopsis plants. Electrical penetration graph data (EPG) were recorded over an 8-hour period. **A** Aphids showed shorter times to first probe on CMV-infected Col-0 and mock-inoculated Ei-2 plants than mock-inoculated Col-0 plants. Aphids probed CMV-infected and mock-inoculated Ei-2 plants at similar times since the beginning of EPG recording. **B** The mean duration of the first probe in minutes is shown. It can be noted that aphids performed shorter probes on CMV-infected Col-0, CMV-infected Ei-2 and mock-inoculated Ei-2 than on mock-inoculated Col-0 plants. Fisher's least significant difference (LSD) test (α=0.05). Error bars denote standard error of the mean. Bars with the same letter within a column are not significantly different ($p > 0.05$), LSD test.

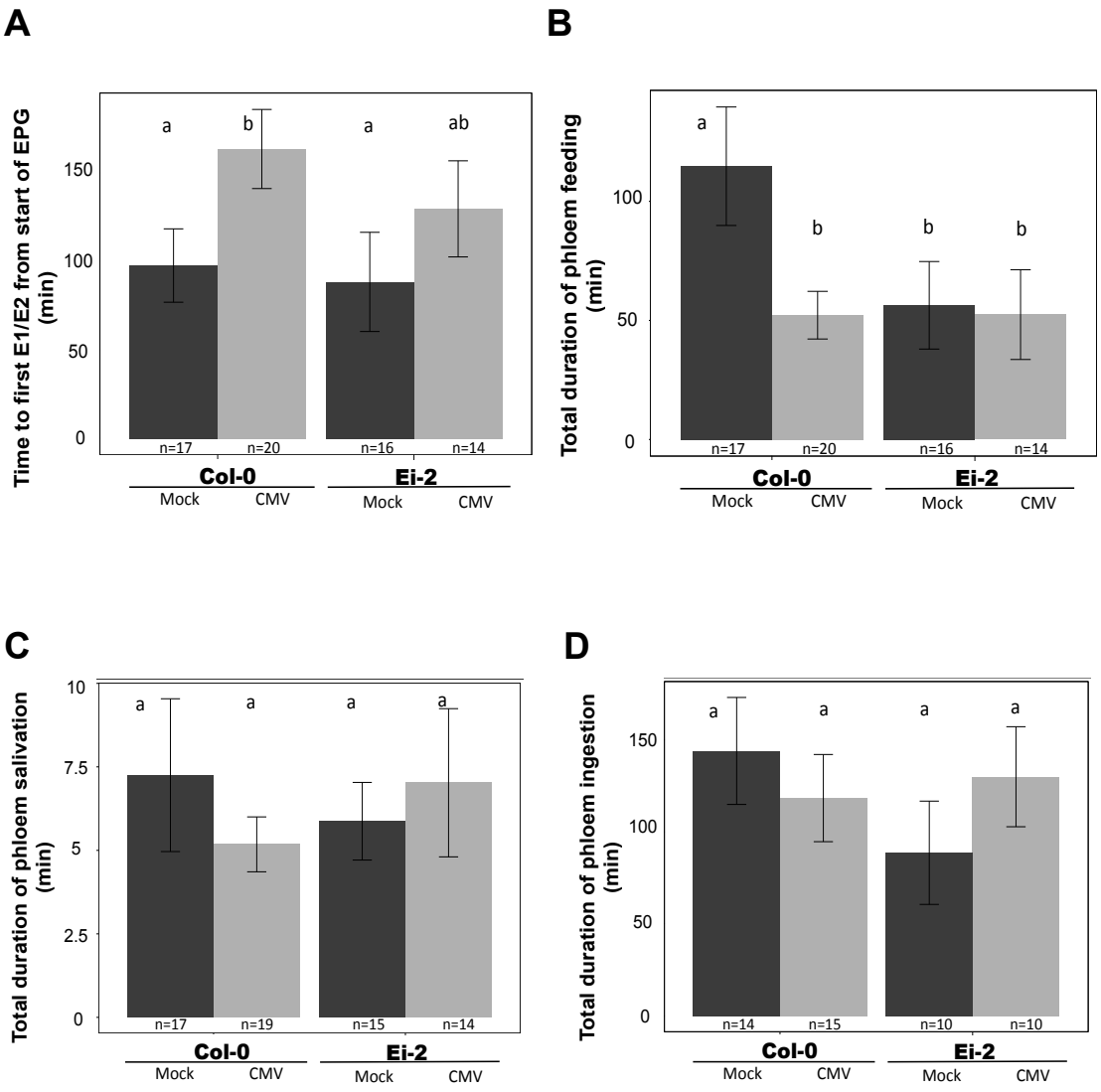


Figure 4.17 EPG: Aphids encounter difficulties in reaching the phloem of CMV-infected Col-0 plants

M. persicae feeding behaviour was evaluated on CMV-infected and mock–inoculated plants of Arabidopsis accessions Col-0 and Ei-2. Electrical penetration graph data were recorded over an 8-hour period. **A** Aphids needed more time to reach phloem on CMV-infected plants compared to mock-inoculated Col-0 plants. No significant differences were found on the time aphids spent to reach E1/E2 on CMV-infected Ei-2 and mock-inoculated Ei-2 plants. **B** Aphids engaged less time on phloem sap ingestion on CMV-infected Col-0, and either mock-inoculated or CMV-Infected Ei-2 plants in comparison with mock-inoculated Col-0 plants. **C-D** No significant differences were observed in the total duration of phloem salivation and phloem ingestion on either mock-inoculated or CMV-infected Col-0 or mock-inoculated or CMV-infected Ei-2 plants. Fisher's least significant difference (LSD) test, (α=0.05). Error bars denote standard error of the mean. Bars with the same letter within a column are not significantly different ($p > 0.05$), LSD test.

4.3 Discussion

4.3.1 CMV infection of Arabidopsis plants induces emission of volatile organic compounds that attract aphids

Herbivorous insects follow a series of steps to find a suitable host plant (Reviewed in Section 1.5.2). Host plant selection is influenced by visual, olfactory and tactile cues (Powell et al., 2006). As observed previously on tobacco (Powell, 1993) and Arabidopsis, aphid starvation increased aphid probing (Figure 4.1), which was critical to assess aphid behaviour on the assays described in this chapter. I consistently found using aphid behaviour assays such as adhesive trap host location assays and Y-tube olfactometry assays, that *M. persicae* was initially attracted to either CMV-infected or TuMV-infected Arabidopsis Col-0 and Ei-2 plants rather than to mock-inoculated plants (Figures 4.9-11).

A previous study reported that VOCs emitted by CMV-infected squash plants had a greater overall concentration of VOCs in headspace and only a few differences in relative concentrations of individual compounds (Mauck et al., 2010). The overall concentration of VOCs emitted by CMV-infected tomato plants was similar to mock-inoculated plants (Groen et al., 2016). I found that CMV-infected Arabidopsis plants emitted qualitatively distinct VOCs compared with mock-inoculated plants from both Col-0 and Ei-2 plants.

The four compounds identified 3-methyl-hexane (Hegde et al., 2011), (*E*)-2hexene-1-ol (Wei and Kang 2011), 2-octanone (Birket et al., 2003) and 3-pentanol (Gols et al., 2011; Song and Ryu, 2013) have been previously identified in the VOCs of plants and are considered to be involved in plant-insect interactions. Interestingly, the four compounds have been found in higher concentrations in plants infested with herbivores (Birket et al., 2003; Gols et al., 2011; Song and Ryu, 2013; Wei and Kang

2011). I found that there is a trend in the variation of the concentration of these compounds and only 3-methyl-hexane and 2-octanone were present at higher concentrations in the headspace of CMV-infected Ei-2 plants compared with CMV-infected Col-0 plants. Further experiments are needed to confirm the identity of these compounds using internal standards. It is important to consider that the input data for the PCA were concentrations of the total amount of volatile organic compounds emitted by the plants. Therefore, the concentrations of these four compounds may not entirely explain the differences observed in the PCA. Knowing the identity of all components of the emitted blend would be useful, however, it has been shown that *A. fabae* respond to the entire blend emitted by *V. faba* plants rather than individual compounds present in the blend (Webster et al., 2008).

Glucosinolates are also recognized to yield volatile compounds which may attract insects (Wittstock et al., 2003). The different glucosinolate profiles reported for these two accessions (Kliebenstein et al., 2001a) might also explain the strong attraction of aphids towards the Ei-2 accession. It should be remembered that although the infected-plants were initially attractive to the aphids, the insects did not settle on them (Section 4.3.2).

4.3.2 CMV infection induces specific aphid performance and settling responses in Col-0 and Ei-2 plants

Aphids were attracted to the plant volatiles emitted by both Col-0 and Ei-2 CMV-infected or TuMV-infected plants. However, aphid settling and aphid performance (growth and fecundity) differed on Col-0 and Ei-2 CMV-infected plants. CMV infection of Col-0 induced aphid resistance whereas it had neutral effects on CMV-infected Ei-2 plants on MRGR and colony size (Figure 4.2). Settling assays showed that aphids consistently preferred to settle on both CMV-infected and mock-inoculated Ei-2 plants rather than CMV-infected and mock-inoculated Col-0 plants (Figure 4.4). Interestingly, Ei-2 is intrinsically more attractive to aphids than Col-0. Taken together,

the results suggest that in different Arabidopsis accessions CMV induces changes in aphid settling behaviour that are accession-specific. The different responses observed in virus-host-vector interactions within Arabidopsis accessions could be explained by the natural variation of glucosinolates and VOCs produced by each accession (Kliebenstein et al., 2001b; Snoeren et al., 2010; Kerwin et al., 2015). In further studies, it would be interesting to test whether these innate differences observed in Arabidopsis also change depending on the type of virus and aphid used e.g. specialist or generalist aphids. My results are in agreement with other studies showing that virus-vector-host interactions can be highly specific in terms of virus strain (Westwood et al., 2013a; Casteel et al., 2014) aphid species (Boquel et al., 2011) and host plant (Eigenbrode et al., 2002; Mauck et al., 2010; Ziebell et al., 2011; Westwood et al., 2013a, 2014; Carmo-Sousa et al., 2014; Tungadi et al., 2017).

4.3.3 CMV infection decreases host quality in Arabidopsis

In the absence of virus infection aphids showed a preference for settling on plants of the glucosinolate mutant line *cyp81 f2-2* rather than on wild-type Col-0 plants. However, CMV infection of *cyp81 f2-2* mutant lines did not encourage aphid settling. Westwood and colleagues (2013a) reported that CMV infection induced a higher concentration of the anti-feedant glucosinolate, 4MI3M, but MRGR assays showed that CMV infection of mutant lines *cyp81f2-1* and *cyp81f2-2* did induce inhibition of growth of aphids confined on these plants. I would have expected that aphids would have preferred to settle on the glucosinolate mutants. One explanation of my results could be that there are some traces of 4MI3M or other toxic compounds present in the glucosinolate mutants that can still cause feeding deterrence. For example, Kettles and colleagues (2013) suggested that the phytoalexin, camalexin, affects aphid performance as aphid fecundity increased on plants with reduced accumulation of camalexin. It was reported that the two independent mutant lines

have extremely low levels of 4MI3M (Clay et al., 2009). An additional explanation of my results could be that having the choice of two plants may facilitate aphid movement to a more suitable host, whereas on MRGR assays aphids are contained on the host plant. In future experiments one could perform assays with completely depleted glucosinolate mutants in indole and/or aliphatic glucosinolates such as *cyp79B2cyp79B3* or *myb28myb29* double mutants (Zhao et al., 2002), in case one type of glucosinolate is more relevant than others in plant-insect interactions. Although only one indole glucosinolate, 4MI3M, has been reported as an aphid deterrent (Kim and Jander, 2007), there could still be some other indole or aliphatic glucosinolates to explore in virus-vector host interactions.

4.3.4 CMV-induced aphid resistance enhances virus transmission

A deceptive attraction to non-persistently transmitted virus infected plants and further aphid deterrence response has been proposed as a mechanism to enhance virus transmission (Mauck et al., 2010; Carmo-Sousa et al., 2014; Groen et al., 2017). It was found that CMV-infection of Arabidopsis plants induces less sustained phloem feeding activities by aphids (Westwood et al., 2013a). I found that aphids had more phloem feeding difficulties on CMV-infected Col-0 than on CMV-infected Ei-2 plants (Figure 4.17). Aphids also exhibited less growth and decreased colony growth on CMV-infected plants (Figure 4.2). Thus, the results suggest that CMV infection in Col-0 plants induces aphid resistance, which might encourage aphid migration away from CMV-infected plants to new hosts. Interestingly, the Arabidopsis accession Ei-2 seems to be a host plant that attracts aphids but is not as susceptible host as Col-0 (Figure 4.2A and Figure 4.16-17). Thus, the intrinsic differences found in Col-0 and Ei-2 indicates that one can exploit natural diversity to manipulate aphid behaviour (See Chapter 5).

Chapter 5 Use of trap plants to attract and sanitise viruliferous aphids to disrupt virus transmission

5.1 Introduction

The previous Chapter described plants from the Arabidopsis accession Ei-2, which are more attractive to *M. persicae* than Col-0 plants (Section 4.2.4). This led me to hypothesise using plants that are more attractive to aphids as trap plants might alter aphid behaviour and disrupt virus transmission. In this Chapter, I investigated how aphid movement and virus transmission can be manipulated by mixing accessions with differences in their intrinsic aphid attractiveness. In Chapter 3, lines of transgenic plants resistant to CMV or TuMV generated in Col-0 and Ei-2 backgrounds were described. I will describe how these virus-resistant lines were used in "microcosm" experiments ("two dimensional" plant arrangements as described in Section 2.7) to explore the concept of sanitising viruliferous aphids in order to inhibit virus transmission. I hypothesised that viruliferous aphids would be rendered non-viruliferous by virus-resistant plants as the virus would be trapped because it will not replicate in a resistant-plant.

In these experiments I refer to the non-trap plants as the "main crop" which are the majority of plants within the microcosm. I designed a microcosm experiment to study virus-host-vector interactions. In the microcosm, I determined the most efficient/detrimental conditions in terms of days post-inoculation of the virus-source plant, number of aphids to transmit the virus and the time post-infestation to reach the highest rate of virus transmission. Thus, I established the conditions at which virus transmission was most efficient in both accessions as a baseline for testing approaches to inhibit virus transmission. I tested three approaches to manipulate aphid behaviour and disrupt virus transmission. These approaches were: (1)

mixtures of plants which varied in their degree of attractiveness to aphids, (2) barriers of virus-resistant trap plants, and (3) different proportions of virus-resistant plants. The approaches were tested with two non-persistently transmitted viruses, CMV and TuMV (Sections 1.2, 1.3.1 and 1.4.1). I further explored whether virus transmission could be disrupted when TuMV-resistant plants and CMV-resistant plants were included in microcosms where the source plants were infected with both viruses.

5.2 Results

5.2.1 How long must virus infection proceed before feeding deterrence is established?

A settling choice test was performed to determine at what time point virus infection begin to influence aphid-host interactions (Section 2.6.7). Aphid settling over 24 hours was assessed at 3, 9 and 21 days post-inoculation. Aphids had an equal preference for settling on virus-infected plants (CMV or TuMV) or mock-inoculated plants at 3 days post-inoculation (Figures 5.1A and B). However, between 9 and 21 days post-inoculation virus-infected plants became less attractive for settlement and aphids settled preferentially on mock-inoculated plants than virus-infected plants (Binomial test, $p < 0.001$).

5.2.2 Aphids emigrate from CMV-infected Arabidopsis plants

Aphid behaviour was assessed in migration experiments to evaluate whether infested CMV-infected plants retain aphids compared to infested mock-inoculated plants (Section 2.6.3, Figure 2.4). As shown in Figure 5.2 fewer aphids remained on CMV-infected "source" plants of either Col-0 or Ei-2 Arabidopsis than on mock-inoculated plants. A small percentage of aphids (37.5%) remained on Col-0 CMV-infected source plants compared to 67% of aphids that stayed on Col-0 mock-inoculated source plants ($\chi^2 = 13.41$, df = 1, $p < 0.001$). Only 26% of aphids stayed on Ei-2 CMV infected plants, whereas 49% remained on mock-inoculated Ei-2

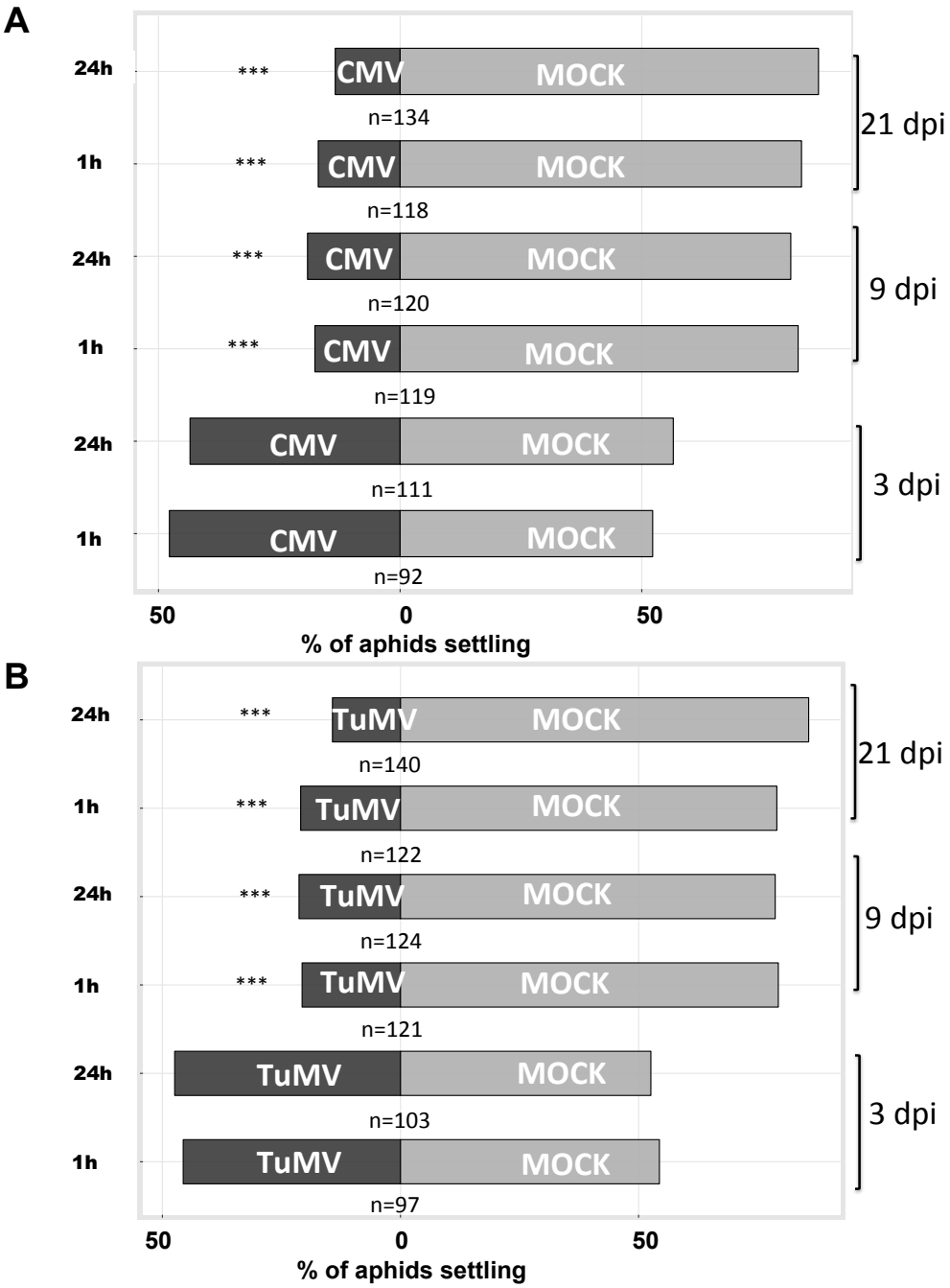


Figure 5.1 Aphid settling on CMV-infected plants varies at different time points following virus inoculation

Aphids were released in arenas containing a mock-inoculated plant and a virus-infected plant at different stages of infection (3, 9, 21 days post inoculation) (Section 2.67, Figure 2.7A). **A** CMV and **B** TuMV show that aphids had equal preference for settling on virus-infected or mock-inoculated plants at 3 dpi, however, at 9 and 21 dpi aphids settled on mock-inoculated plants. The graphs represent experiments performed on Arabidopsis Ei-2 plants. Each time point was performed independently. A total of 10 arenas and 30 aphids per arena were used for each experiment. After 1 hour and 24 hours of aphid release, aphids were counted on individual plants of each arena. Asterisks denote statistically significant differences based on binomial test, *** $p < 0.001$.

plants (χ^2 = 7.78, df = 1, p < 0.01). The results suggest that in both Arabidopsis accessions CMV infection encourages aphids to migrate towards uninfected plants.

5.2.3 Aphid movement dynamics are altered when "source" and "line" plants belong to different accessions

I have described in previous sections that Col-0 and Ei-2 differ in their attractiveness to the aphid, *M. persicae.* I wanted to explore whether those differences could be exploited to modify the dynamics of aphid migration and virus transmission. For that, I used the same set-up as described in Section 5.2.4, but using Col-0 source plants for lines of Ei-2 plants, and Ei-2 source plants for lines of Col-0 plants. I found that Col-0 as a CMV source plant adjacent to Ei-2 plants (more attractive than Col-0) changed aphid migration (Figure 5.3). As shown in Figure 5.3A an extremely reduced percentage of aphids 14% remained on the source CMV-infected Col-0 plants compared with 57% of aphids found on mock-inoculated Col-0 plants (χ^2= 26.22, df = 1, p < 0.001).

In contrast, the alternative combination (CMV-infected Ei-2 source plant adjacent to Col-0 plants) displayed similar percentages of aphids retained on source plants as follows, 21% of aphids were found on CMV-infected Ei-2 source plant and 28% of aphids were found on mock-inoculated Ei-2 source plant (χ^2 = 1.06, df = 1, p = 0.30) (Figure 5.3B). These results suggest that mixture of hosts can be used to manipulate aphid behaviour.

Additionally, it can be noted that the highest number of infected plants were found on lines where Col-0 was the CMV-source plant adjacent to Ei-2 susceptible plants (Table 5.1). A summary diagram showing the extent of virus infection as well as the proportion of plants infected for the four treatments assessed in emigration experiments is shown in Figure 5.4. However, very small proportion of infection occurred and the results were not statistically significant (Table 5.1 and Figure 5.4)

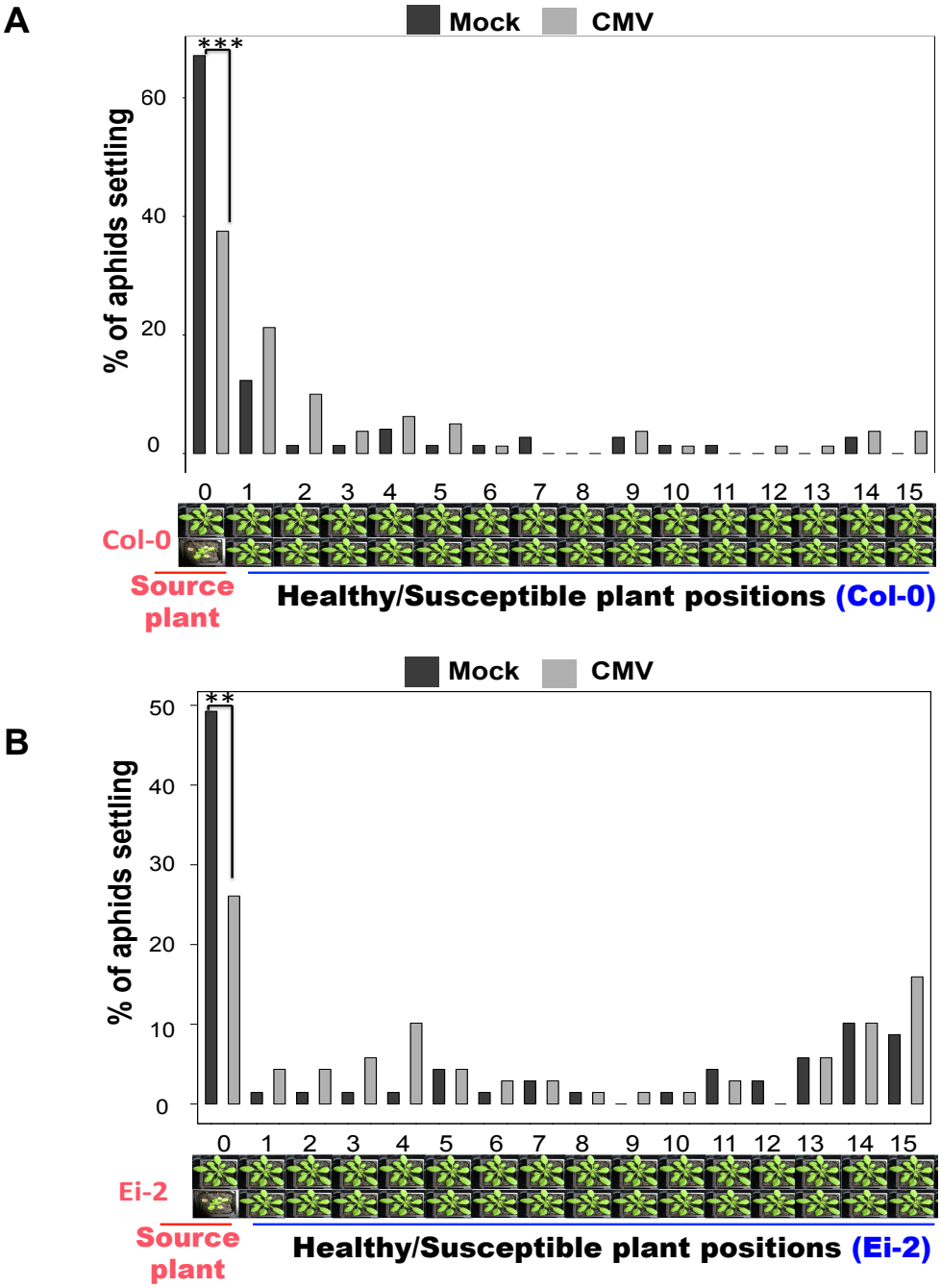


Figure 5.2 Aphids migrate away from CMV-infected plants
Each experiment consisted of 14 lines of plants with either CMV-infected source plants or mock-inoculated plants in the "zero" position. Six 7-day old aphids (*M. persicae)* were placed on each CMV-infected or mock-inoculated source plants. After 24 hours, aphids were counted on each plant along the lines and insecticide was applied to stop further transmission. The plants were kept for two weeks to assess virus infection. In these set of experiments the same Arabidopsis accession was used as a source and healthy/susceptible plants. A small proportion of aphids were found on CMV-infected source plants of either accession after 24 hours of infestation. **A** shows that 37% of aphids (n = 80) stayed on CMV-infected Col-0 plant whereas 67% (n = 73) remained on mock-inoculated Col-0 plants. (χ^2 = 13.41, df = 1, *** p < 0.001) **B** indicates that 26% of aphids (n = 69) remained on CMV-infected Ei-2 plants than 49% (n = 67) on mock-inoculated plants (χ^2 = 7.78, df = 1, **p <0.01). Only the proportion of aphids found on source plants were used for statistical test.

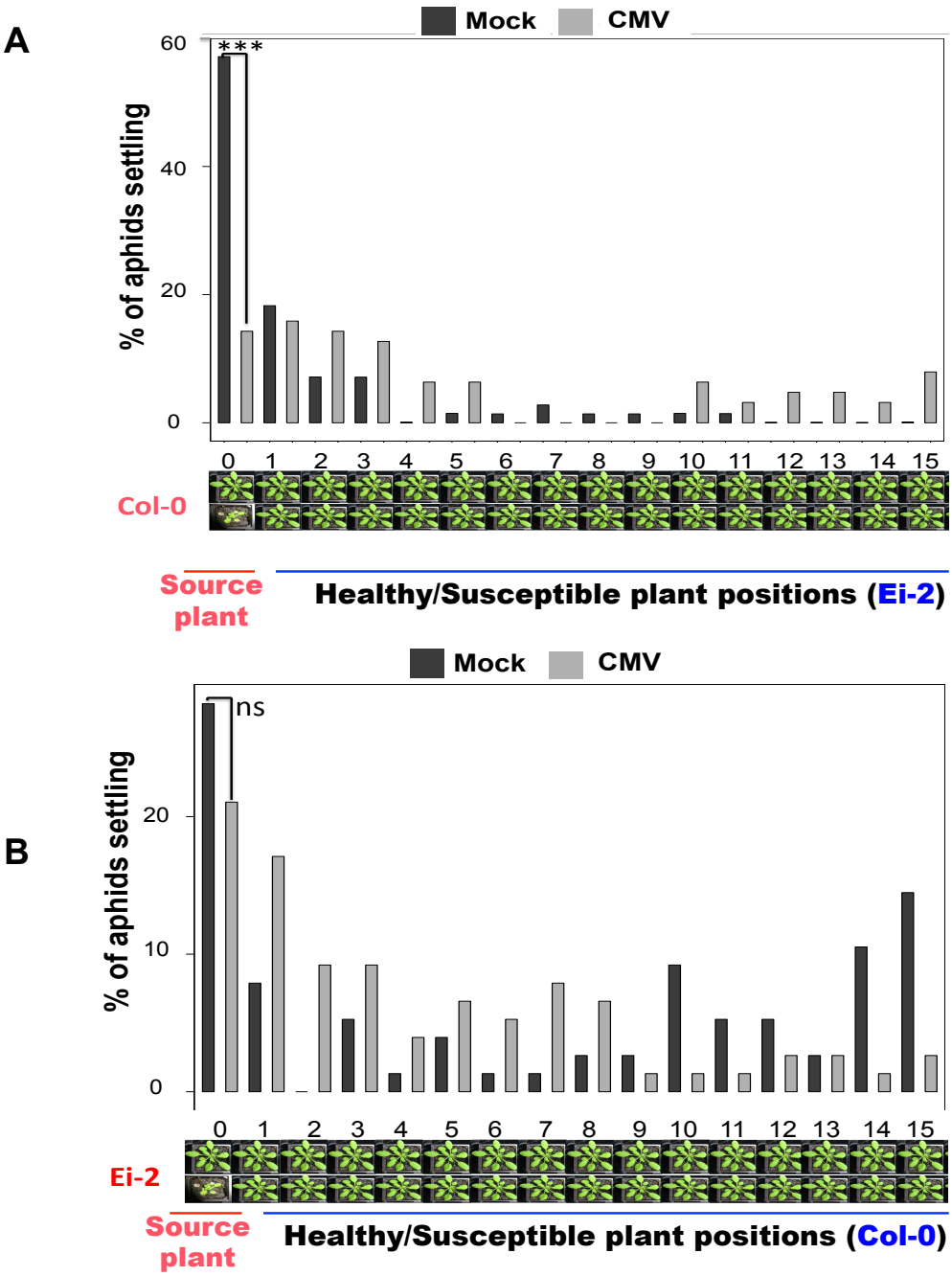


Figure 5.3 Aphid migration dynamics from virus-infected source plants is altered in "mixed" lines

Each experiment consisted of 14 lines of plants with either CMV-infected source plants or mock-inoculated plants in the "zero" position and followed by 15 plants of the other accession (i.e Col-0 source plants with Ei-2 susceptible plants and Ei-2 source plant with Col-0 susceptible plants). Six (7-day old) aphids were placed on each CMV-infected and mock-inoculated source plant. After 24 hours aphids were counted on each plant along the lines and insecticide applied to further transmission. The plants were kept for two weeks to assess virus infection. **A** shows that 14% of aphids (n = 63) stayed on CMV-infected Col-0 plant whereas 57% (n = 72) remained on mock-inoculated Ei-2 plants. (χ^2= 26.22, df = 1, *** p < 0.001) **B** indicates similar proportions of aphids 21% (n = 76) remained on CMV-infected Ei-2 plants and 28% (n = 78) on mock inoculated Ei-2 plants (χ^2 = 1.06, df = 1, p=0.30). Only the proportion of aphids found on source plants were used for statistical test.

Table 5.1 Proportion of plants infected in aphid migration assays

Source plant	Susceptible plants	Plants infected	Total number of susceptible plants	Proportion of plants infected	X^2
Ei-2	**Col-0**	**4**	**210**	**2%**	
	Ei-2	**1**	**210**	**0.50%**	**ns**
Col-0	**Ei-2**	**12**	**210**	**6%**	
	Col-0	**6**	**210**	**3%**	**ns**

The proportion of plants infected found on each aphid migration assay/treatment (Figures 5.2 and 5.3) is shown. Two weeks after aphid movement assessment, the number of plants showing CMV symptoms along the lines was counted (virus infection was confirmed with ELISA). Each experiment consisted of 14 lines with 15 susceptible plants, (i.e. 210 susceptible plants per experiment). The differences in the proportions of plants infected were not statistically significant (Chi square test).

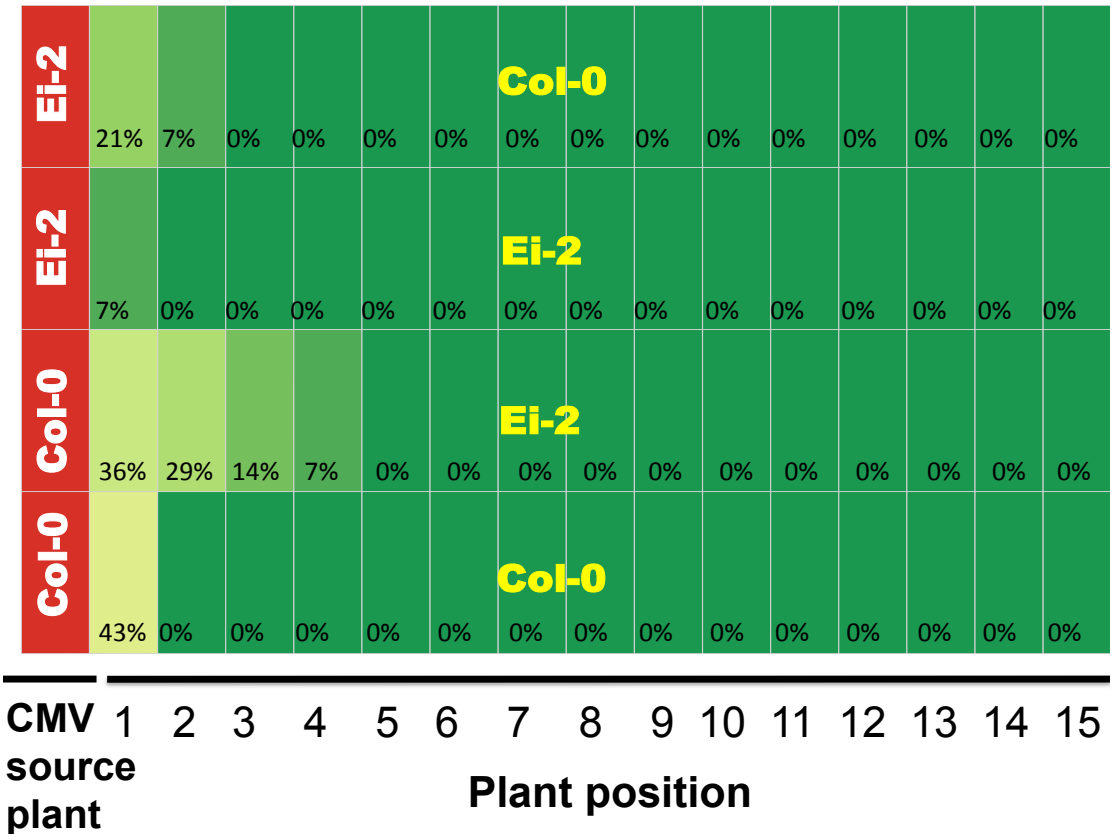


Figure 5.4 Summary diagram of aphid-mediated CMV transmission on aphid migration assays

A migration assay consisted of 14 lines of 16 plants. The CMV-infected (10 days post inoculation) source plants were placed next to 15 susceptible/healthy plants. After two weeks of aphid migration from CMV-infected source plants, the spread of virus transmission by aphids was evaluated. The number of plants infected on each plant position along the line was estimated (i.e total number of plants found at each position along the line). The diagram shows the plant position and proportion of plants infected found in the four treatments assessed in the migration assays showed in Figures 4.18 and 4.19. The first column in red represents the Arabidopsis accession CMV-source plant. The proportion of plants infected at each position along the line was assigned with a colour that indicates the proportion of plants infected with CMV after aphid transmission. Colour gradient in this heat map correlates with the proportion of plants infected as follows red (high), yellow (intermediate) and green (no infection). The proportion of infected plants at each plant position was not significantly different (Chi square test).

It can be noted in the diagram that Col-0 as a virus infected source plant adjacent to Ei-2 plants not only had the higher proportion of infected plants but also virus infection spreads further along the line compared with the non-mixed lines. Although the results suggest that aphid behaviour was altered, virus spread was not significantly different when Col-0 and Ei-2 were mixed. Taken together mixtures of Col-0 and Ei-2 can be used to manipulate aphid behaviour and potentially virus transmission.

5.2.4 Col-0 and Ei-2 are similarly susceptible to CMV and TuMV

The contrasting responses of aphids observed on CMV infected Col-0 and Ei-2 plants might be a result of virus titre. To evaluate whether these accessions exhibit a different accumulation of CMV, I analysed the virus titre by ELISA (Section 2.2.12). The results shown in Figure 5.5 indicate that plants of both accessions exhibit a similar degree of susceptibility to CMV infection. In addition, I evaluated the titre of another non-persistently transmitted virus, TuMV. Both accessions show similar susceptibility to TuMV.

5.2.5 Virus transmission is most efficient at 10 days post-inoculation

Virus transmission was assessed in 3x3 arrays of plants where the virus-infected source plant was placed in the centre of arrays and the rest of the plants were uninfected plants (Section 2.7 and Figure 2.9). Transmission was evaluated at different days post-inoculation (3, 5, 7, 10, 14 dpi). I found that transmission efficiency of CMV on Arabidopsis accessions Col-0 and Ei-2 varied according to the duration of virus infection (Figure 5.6). At early time points of infection (3 dpi), 38% of Col-0 plants were CMV-infected compared to 8% of Ei-2 plants ($\chi^2 = 5.78$, df=1, **$p < 0.01$). The accessions exhibited the most similar percentages of infected plants when the virus-infected source plant was at 10 dpi. At 10 dpi, the maximum percentages of plants infected were 46% on Col-0 and 42% on Ei-2.

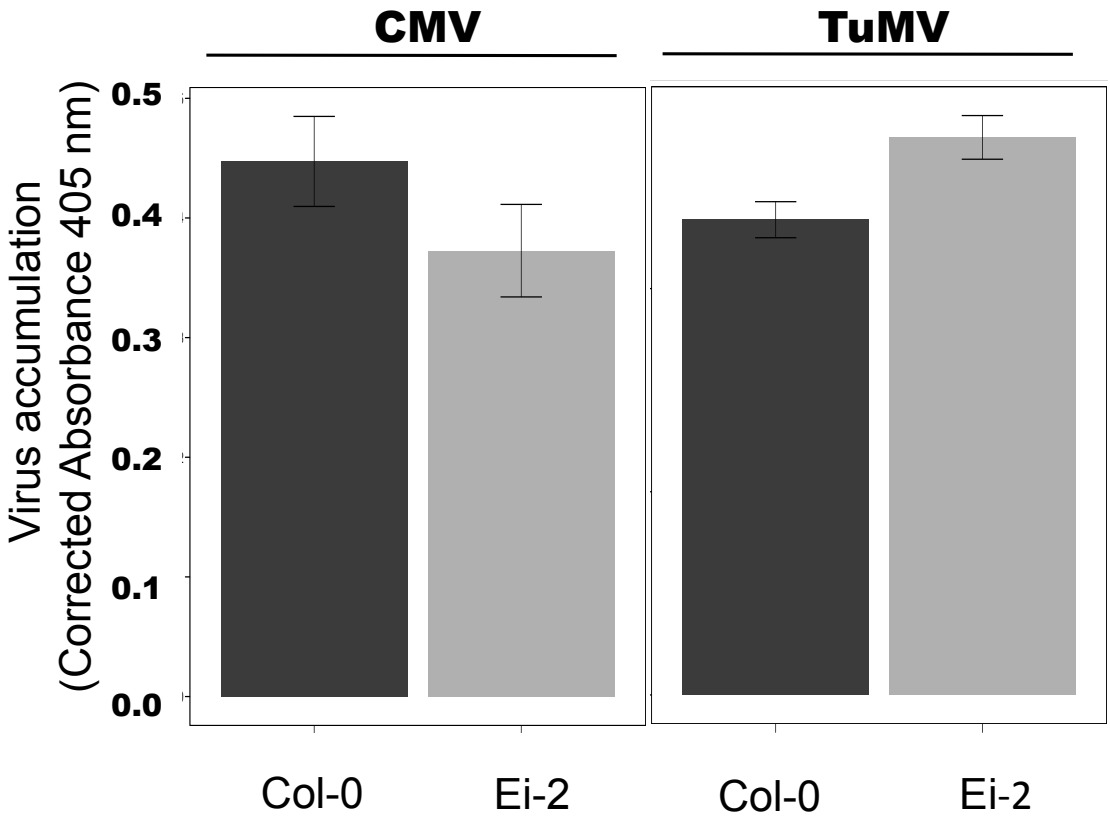


Figure 5.5 Arabidopsis accessions Col-0 and Ei-2 are equally susceptible to CMV and TuMV

Using ELISA virus titre was evaluated on Col-0 and Ei-2 infected with either CMV or TuMV. Plants of Col-0 and Ei-2 Arabidopsis accessions accumulate similar titres of both viruses (n = 15). The background A_{405} values of mock-inoculated plants were subtracted to obtain the corrected A_{405} values displayed. No significant differences in titre were found between the accessions (Student's *t* test). Error bars denote standard error of the mean.

Overall, in both accessions the efficiency of virus transmission increased as virus infection spread systematically (3-7 dpi), reached the highest point at 10 dpi and decreased by 14 dpi.

5.2.6 Optimizing conditions for CMV transmission

As shown in Figure 5.7, Col-0 and Ei-2 exhibited similar percentages of virus-infected plants at different hours post-infestation (1, 2, 3, 6, 9 and 24 hours). At 24-hours post- infestation the percentage of CMV-infected plants in both accessions were the most similar with 46% of Col-0 and 50% of Ei-2 plants found to be virus-infected. CMV-infected source plants were infested with different aphid numbers (6, 15, 30 and 60 aphids per source plant) to determine the optimal aphid density to achieve similar transmission on Col-0 and Ei-2 Arabidopsis accessions (Figure 5.8). Ei-2 CMV-infected source plants infested with 6 aphids exhibited the lowest percentage of CMV-infected plants, 4%, compared with 29% of CMV-infected plants found in Col-0 plants (χ^2 = 5.4, df=1, * $p < 0.05$.). CMV source plants of both Col-0 and Ei-2 infested with 30 aphids showed the most similar percentages of virus-infected plants, 71% and 67%, respectively. When CMV source plants were infested with 60 aphids, the percentage of plants infected after virus transmission by aphids did not increase any higher (Figure 5.8).

5.2.7 Aphids are lured to “trap” plants in microcosm experiments

Microcosm experiments were performed as described in Section 2.7. As depicted in Figures 5.9C and 5.10C, the terms: first layer and second layer were used to describe the location of trap plants within the microcosm. The “trap” plants were placed in three different layouts within the microcosm as follows: (i) trap plants on first layer of plants, (ii) trap plants on second layer of plants and (iii) trap plants on both layers of plants. The distribution of aphids on each layer was compared with the control, which was a microcosm with no “trap” plants. Figure 5.9 shows arrays in which Col-0 was the “main crop” and Ei-2 was the trap plant.

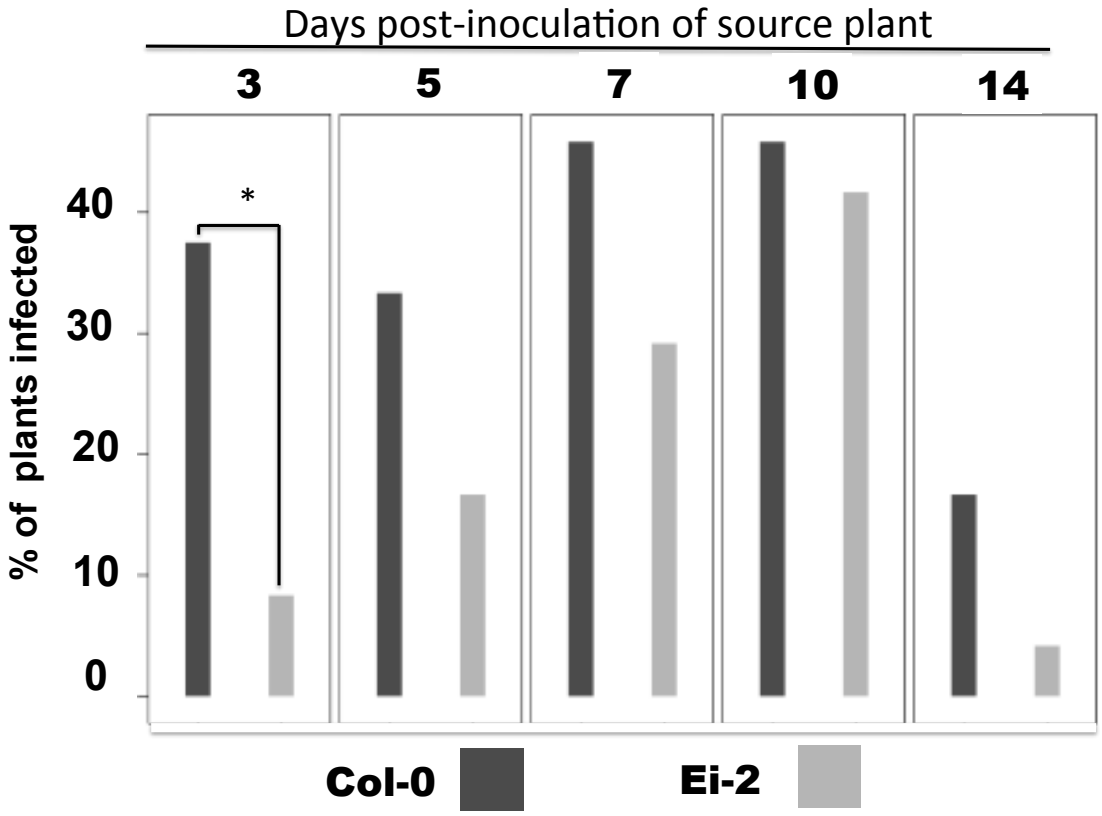


Figure 5.6 Aphid transmission from CMV-infected Arabidopsis Col-0 and Ei-2 to surrounding plants is the most efficient when the source plant has been infected for 10 days

CMV-infected source plants were infested with 30 aphids and placed in the centre of 3x3 arrays of plants (n = 3) (Figure 2.3). After 24 hours of aphid infestation, insecticide was applied. Two weeks post-infestation, the number of virus-infected plants was assessed. The CMV-infected source plant was used at 3, 5, 7, 10, 14 days post-inoculation. Each time point (days post-inoculation) was tested independently. Col-0 and Ei-2 accessions were observed at the same time to compare the efficiency of virus transmission. $\chi^2 = 5.78$, df=1, **p < 0.01.

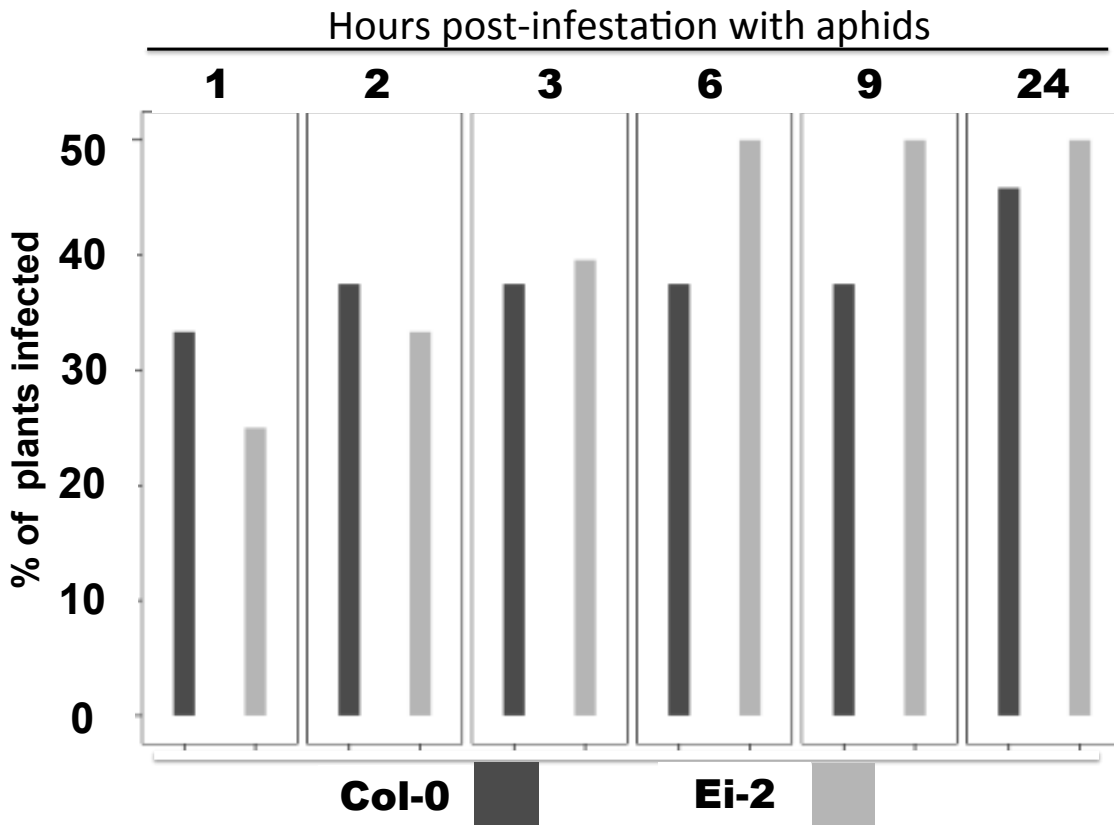


Figure 5.7 Aphids transmit CMV similarly from CMV-infected Col-0 and Ei-2 plants at different time points post-infestation

CMV-infected source plants (10 dpi) were infested with 30 aphids and placed in the centre of a 3x3 array of either Col-0 or Ei-2 plants (n = 3) (Figure 2.3). After 1, 2, 3, 6, 9 or 24 hours of aphid infestation, insecticide was applied to the plants. Two weeks post-infestation, the number of virus-infected plants was assessed. Each time point (hours post- infestation) was tested independently. Col-0 and Ei-2 accessions were observed at the same time to compare the efficiency of virus transmission. No significant differences were found between both accessions at each time point based on Chi-square test.

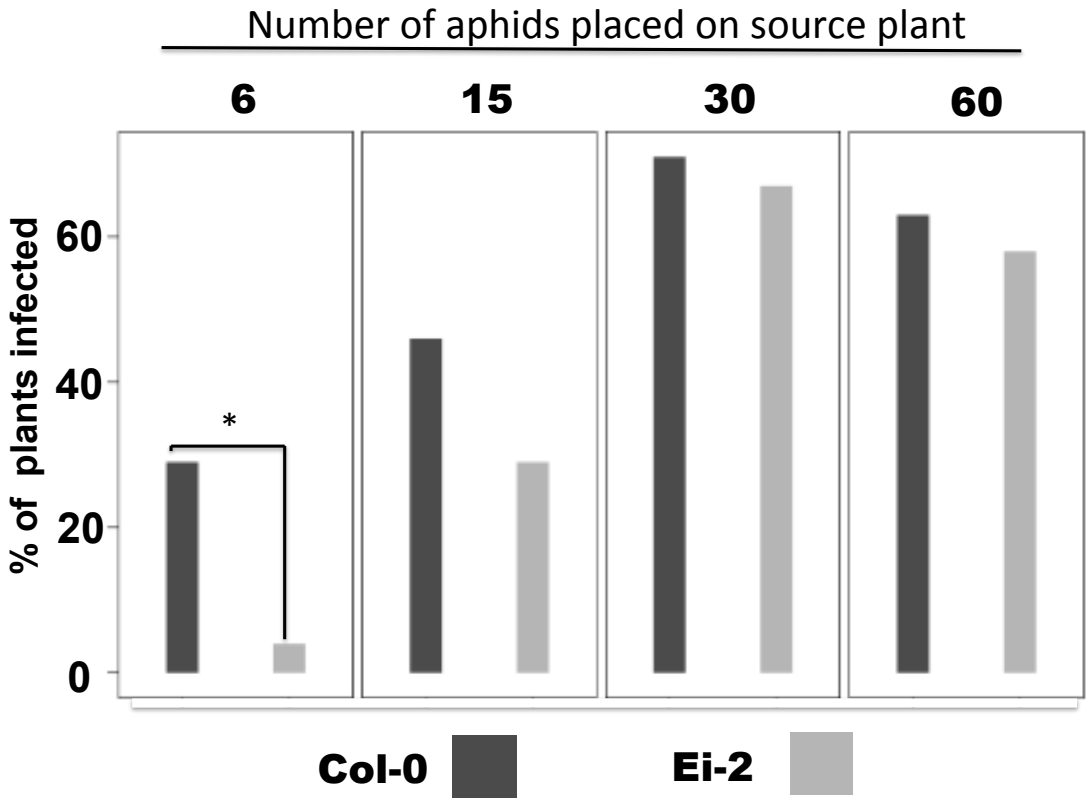


Figure 5.8 CMV transmission is most efficient when the virus source plant is infested with 30 aphids

CMV-infected source plants were infested with 6, 15, 30 or 60 aphids and placed in the centre of 3x3 arrays of either Col-0 or Ei-2 plants (n = 3) (Figure 2.3). After 24 hours of aphid infestation, insecticide was applied to the plants. Two weeks post-infestation, the number of virus-infected plants was assessed. Col-0 and Ei-2 accessions were observed at the same time to compare the efficiency of virus transmission. $\chi^2 = 5.4$, df=1, *$p < 0.05$.

Out of the three layouts tested in microcosms containing Col-0 source plants, the layout where Ei-2 plants were located in the first layer of plants trapped more aphids (56%) than the first layer of plants in the control (37%) (GLM, $p < 0.01$) (Figure 5.9A). As a result, in this layout a reduced percentage of aphids were found on the second layer of plants (37%) compared with percentage of aphids found (61%) on the second layer of plants in the control (GLM, $p < 0.001$). These results showed that Ei-2 plants placed on the first layer of a Col-0 microcosm lured and trapped aphids. Although the aphids were decoyed to Ei-2 plants, the percentage of virus infected plants after virus transmission by aphids was similar to the control (Figure 5.9B).

The layouts described above were also tested in microcosms containing Ei-2 plants where Col-0 was the “trap” plant. I again found that a first layer of trap plants altered aphid settling (Figure 5.10 A). These results are explained in two observations. First, 37% of aphids were found on the first layer of Col-0 plants compared with 20% found in the control (GLM, $p <0.01$). It was also noted that 45% of aphids were found on the second layer of Ei-2 plants, compared with 76% of aphids found in the control (GLM, $p < 0.001$). The percentage of infected plants was still similar in most of the layouts evaluated (Figure 5.10B). However, the layout where Col-0 plants were placed on the second layer had only 5% of plants infected compared with 15% of plants infected on the second layer of plants in the control (GLM, $p <0.05$). Taken together, the results suggest that I succeeded in manipulating dispersal of aphids by placing “trap” plants microcosm experiments. I will refer to this first layer of plants as a barrier in the experiments described in Section 5.2.10.

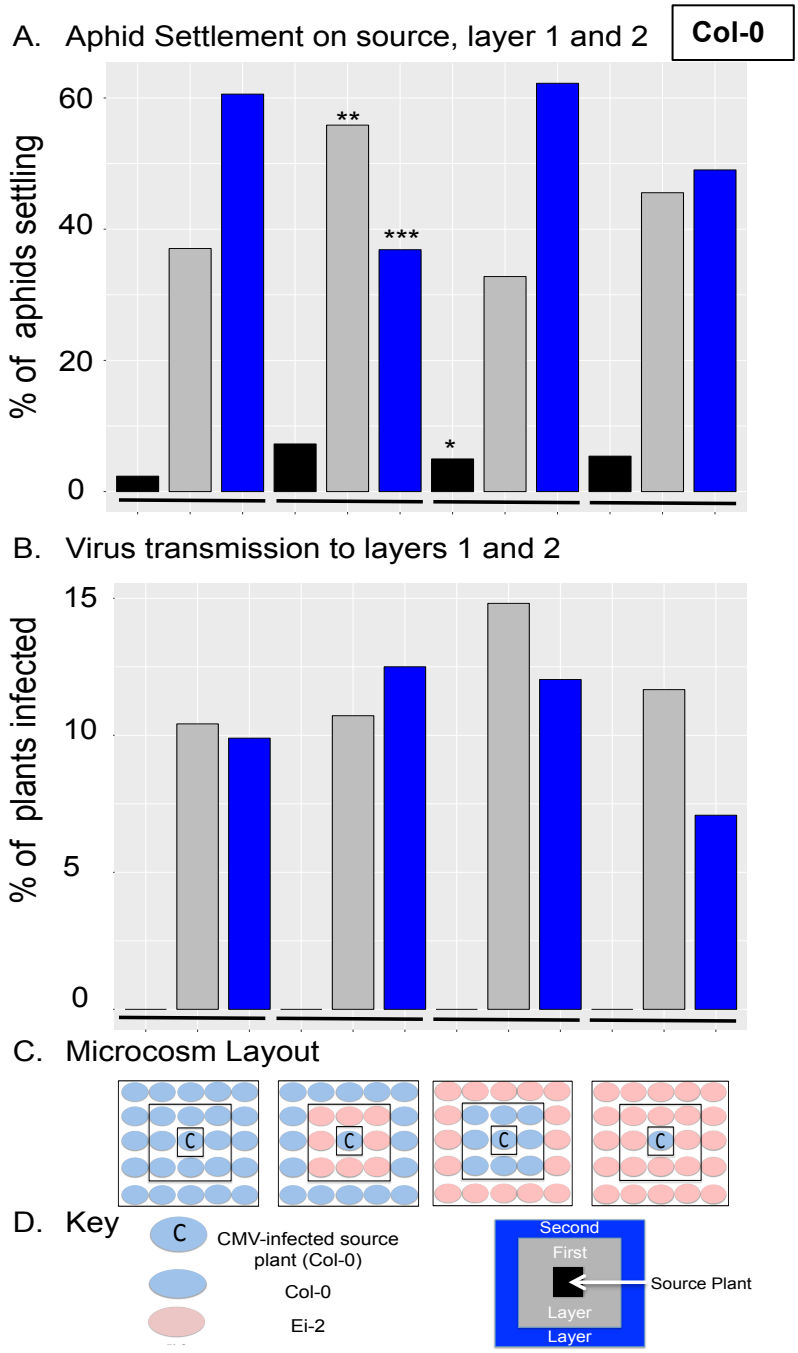


Figure 5.9 Aphids are trapped by a barrier of Ei-2 plants in Col-0 microcosms
CMV-infected Arabidopsis Col-0 (10 dpi) source plants (infested with 30 aphids) were placed in the centre of 5x5 arrays of plants (n = 8) (Section 2.7, Figure 2.9). Aphid movement was assessed by counting aphids on each layer of plants. After counting of aphids, insecticide was applied and arrays were kept for 2 weeks until virus symptoms appeared. In these experiments **Col-0** was the "main crop" and Ei-2 the trap plant. **A** shows the percentage of aphids found on the source plant (black), first layer of plants (grey) and second layer of plants (blue). **B** shows the percentage of CMV-infected plants found on the first layer (grey) and second layer (blue) of plants. **C** shows a diagram of the layouts used in the 5x5 arrays. Light blue circles represent Col-0 plants and pink represent Ei-2 plants. Significant differences were determined by a generalised linear model *p < 0.05, **p < 0.01, ***p < 0.001. The percentages of aphids and infected plants found on each layer per treatment were compared with those found on each layer of the control.

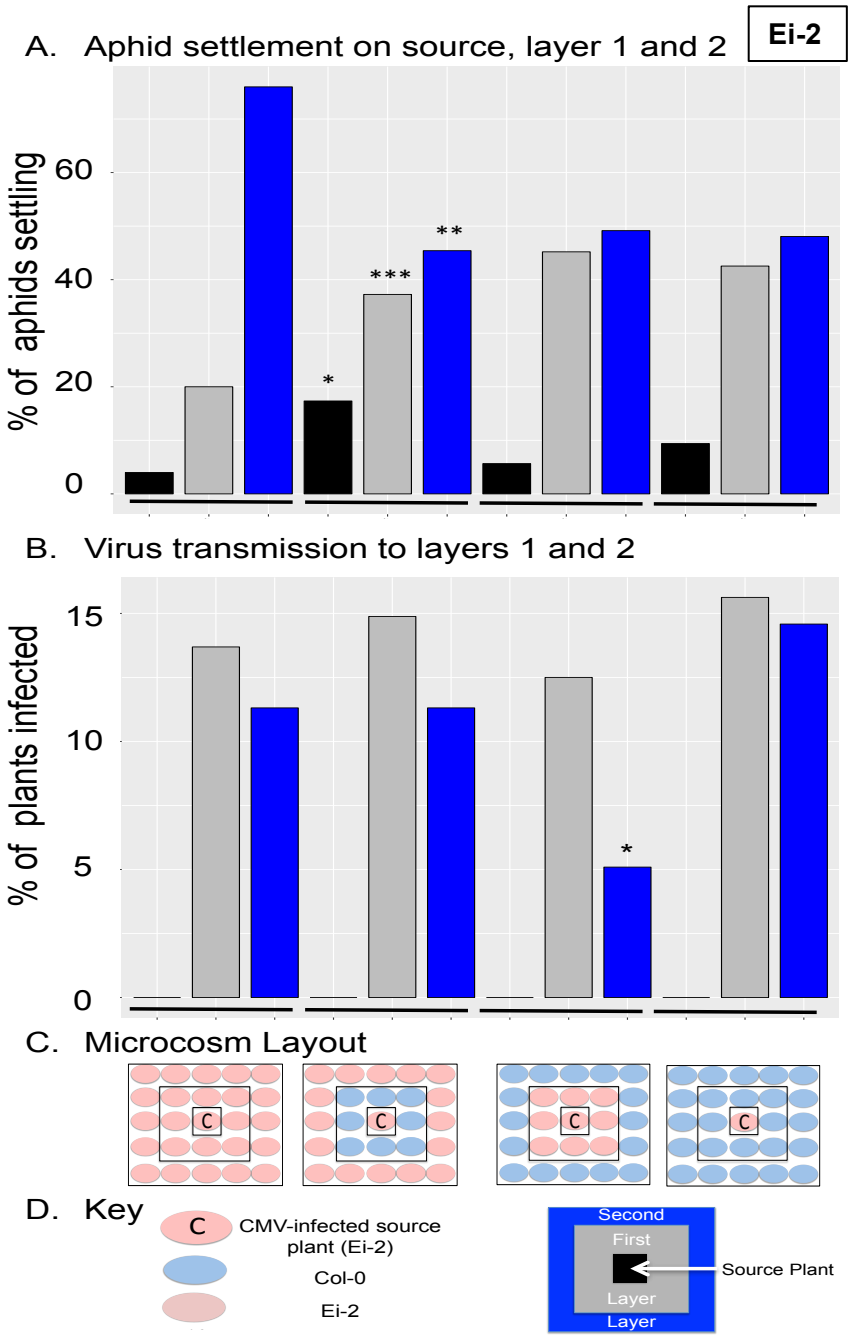


Figure 5.10 Aphids are lured to attractive Ei-2 plants in Ei-2 microcosms

CMV-infected (10 dpi) source plants (infested with 30 aphids) were placed in the centre of 5x5 arrays of plants (n = 8) (Figure 2.10). Aphid movement was assessed by counting aphids on each layer of plants. After counting of aphids, insecticide was applied and arrays were kept for 2 weeks until virus symptoms appeared. In these experiments **Ei-2** was the main crop and Col-0 the trap plant. **A** shows the percentage of aphids found on the source plant (black), first layer of plants (grey) and second layer of plants (blue). **B** shows the percentage of CMV-infected plants found the first layer (grey) and second layer (blue) of plants. **C** shows a diagram of the layouts used in the 5x5 arrays. Significant differences were determined by a generalised linear model *p < 0.05, **p < 0.01, ***p < 0.001. The percentages of aphids and infected plants found on each layer per treatment were compared with those found on each layer of the control.

5.2.8 A small proportion of virus-resistant plants within a population of non-resistant plants can disrupt virus transmission

The TuMV-resistant line Col-0 HP-PIPO 5.53 conferred complete resistance to TuMV (Table 3.3, Chapter 3). Therefore, I used Col-0 HP-PIPO 5.53 to test whether a small proportion of highly resistant-plants randomly distributed (Section 2.7) in a microcosm would reduce virus transmission within a population of plants dominated by susceptible hosts. As shown in Figure 5.11, 33% of TuMV-resistant plants randomly distributed in a microcosm of non-resistant plants was sufficient to decrease the infection rate by 50% compared with the control, which was a microcosm with non-TuMV-resistant plants (GLM, $p < 0.01$). As expected an array with 100% virus-resistant plants stopped virus transmission from a virus-infected source plant (GLM, $p < 0.001$) (Figure 5.11). This experiment shows that small proportions of virus-resistant plants randomly distributed within a population of non-resistant plants can reduce the rate of virus infection.

5.2.9 An aphid-deterrent and highly CMV-resistant plant line reduces virus transmission

One of the CMV-resistant lines, Col-0 ami-SD-3 1.12, was highly resistant to CMV and, unexpectedly, deterrent to *M. persicae* (Section 3.2.7, Figure 3.12 and Table 3.3). I tested whether including various proportions (randomly distributed) of this particular line in microcosm experiments would affect virus transmission (Section 2.7, Figure 2.10). I found that even using only a very low proportion of this line, 0.17, was effective to reduce the number of infected plants by 55% in microcosm experiments (GLM, $p < 0.01$) (Figure 5.12). As the proportion of plants of Col-0 ami-SD-3 1.12 line increased in the arrangements, the number of infected plants decreased compared with microcosms with non-virus-resistant plants. I found that 33% of CMV-resistant plants reduced the rate of virus infection by 60% (GLM, $p < 0.01$) (Figure 5.12).

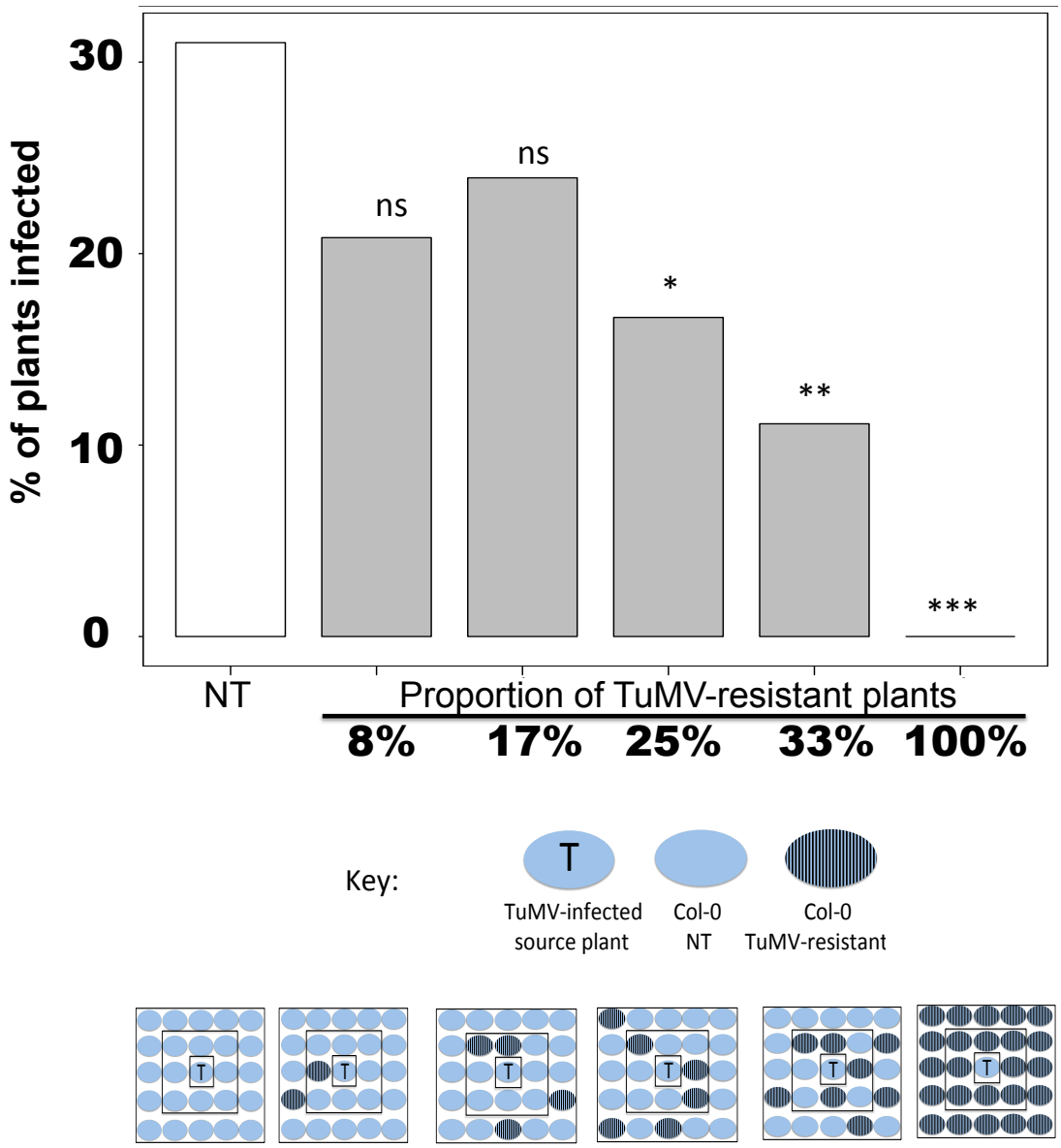


Figure 5.11 Small proportions of TuMV-resistant plants within a population of susceptible plants are sufficient to reduce virus transmission

TuMV infected (10 dpi) source plants (infested with 30 aphids) were placed in the centres of 5x5 arrays of plants (n = 8) (Section 2.7, Figure 2.10). Various proportions (8%, 17%, 25%, 33% and 100%) of TuMV-resistant plants (Line HP-PIPO 5.53) were randomly placed in the microcosms in which the remainder of plants were non-transgenic (NT). After 24 hours of aphid infestation, insecticide was applied. Arrays of NT plants were used as controls. Arrays of plants were kept for 2 weeks until virus symptoms appeared. Significant differences were determined by a generalised linear model *p < 0.05, **p < 0.01, ***p < 0.001.The percentage of plants infected found in each treatment was compared with percentage of infected plants found in the control.

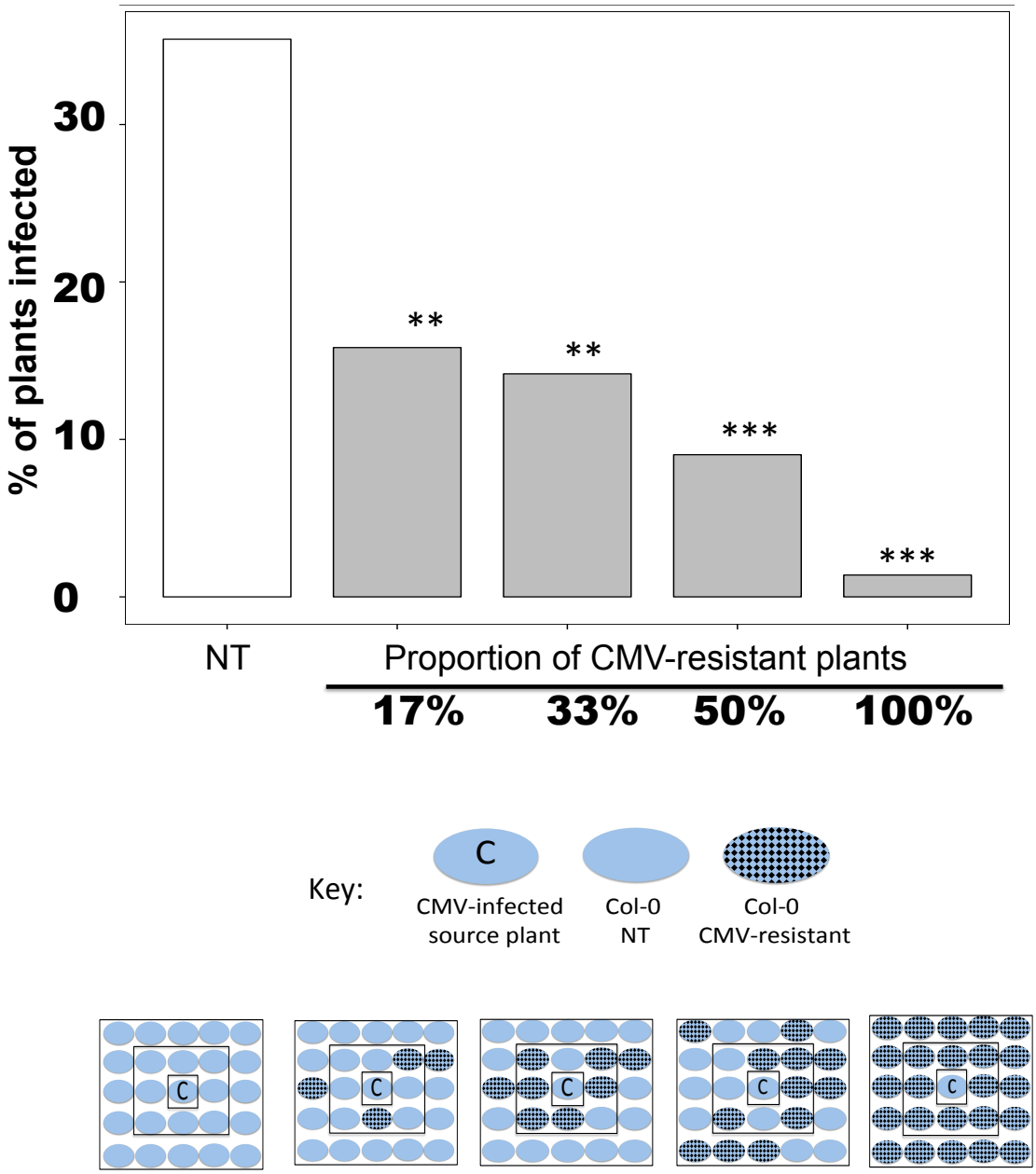


Figure 5.12 A small proportion of a virus-resistant and aphid deterrent line occurring randomly decreases CMV transmission

CMV-infected (10 dpi) source plants (infested with 30 aphids) were placed in the centres of 5x5 arrays of plants (n = 8) (Section 2.7, Figure 2.10). Various proportions (17%, 33%, 50% and 100%) of the line Col-0 ami-SD3 1.12 CMV-resistant and aphids deterrent plants (Section 3.2.9) were randomly placed in the microcosms in which the remaining plants were non-transgenic (NT). After 24 hours of aphid infestation, insecticide was applied. Arrays of plants were kept for 2 weeks until virus symptoms appeared. Arrays of NT only plants were used as controls. Significant differences were determined by a generalised linear model *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. The percentage of plants infected found in each treatment was compared with percentage of infected plants found in the control.

The occurrence of 50% and 100% of CMV-resistant plants in a population of susceptible plants reduced the rate of virus infection by 75% and 96%, respectively (GLM, $p < 0.001$) (Figure 5.12). These results again suggest that small proportions of virus-resistant plants (randomly distributed) can reduce virus infection in microcosm experiments.

5.2.10 Virus-resistant plants enhance the disruption of virus transmission

In Section 5.2.7 it was shown that aphids could be lured to trap plants used as barriers (Figures 5.9A and 5.10A) and that including 33% of virus-resistant plants randomly distributed within microcosms disrupts virus transmission (Figures 5.11 and 5.12). I further investigated the use of both virus-resistant and trap plants to test whether the reduction of virus transmission could be improved. For that, I evaluated two types of arrangement as follows: (a) barrier of virus-resistant trap plants and (b) 33% of aphid attractive and virus-resistant plants distributed randomly.

The results shown in Figures 5.13 and 5.14 are from experiments performed with TuMV-resistant plants in Col-0 and Ei-2 microcosms, respectively. Regarding aphid movement, Figure 5.13A shows that 48% and 47% of aphids settled on barriers of either TuMV-resistant or susceptible Ei-2 plants compared with 28% of aphids settled on the first layer of solely Col-0 plants (GLM, $p < 0.01$). In terms of virus transmission, Figure 5.13B indicates that barriers of TuMV-resistant and non-resistant Ei-2 plants reduced TuMV transmission to the second layer to 4.6% and 4.1% compared with 15% in the control (GLM, $p < 0.001$). Barriers of TuMV-resistant Col-0 plants also reduced TuMV transmission and noticeably the barrier of these highly virus-resistant plant reduced TuMV transmission to 0% as no virus-infected plants were found in the barriers compared with 16% infected plants found in the first layer in the control (GLM, $p < 0.001$).

Figure 5.13B also shows that 33% of TuMV-resistant Col-0 plants randomly distributed in microcosms of Col-0 plants reduced the rate of virus infection to 8% and 7.7% in the first and second layer compared with the 16% and 15% in the control where only susceptible Col-0 plants were present in the microcosm (GLM, $p < 0.001$). However, 33% occurrence of TuMV-resistant Ei-2 plants did not significantly reduce virus transmission

Barriers and 33% of randomly distributed TuMV-resistant plants were also evaluated in microcosm of Ei-2 plants. Figure 5.14A shows that barriers of both TuMV-resistant and susceptible Col-0 plants only trapped 5% (GLM, $p < 0.01$) and 16% (GLM, $p < 0.001$) of aphids compared with 23% of aphids found on the first layer of plants in the control. As shown in Figure 5.14B, the barrier of TuMV-resistant Col-0 plants stopped virus infection to 0% (GLM, $p < 0.001$) compared with 19.6% of plants infected in first layer of the control. Interestingly, the barrier of TuMV-resistant Ei-2 plants reduced virus infection to 1.2% and 2.4% in the first and the second layer compared with 19.6% and 15% of plants infected in the first and second layer of the control (GLM, $p < 0.001$). As expected the barrier of susceptible Col-0 plants did not significantly reduced the number of TuMV-infected plants. Similar to the results observed in microcosm of Col-0 plants, I also found that randomized mixtures of 33% of TuMV-resistant plants within susceptible Ei-2 plants significantly reduced TuMV transmission (Figure 5.14B). TuMV transmission in the first layer was reduced to 9% and 7% (GLM, $p < 0.05$) when TuMV-resistant Col-0 or TuMV-resistant Ei-2 plants were randomly mixed within populations of Ei-2 plants (Figure 5.14B) compared with 19% of infected plants found in the first layer of the control. In addition, TuMV transmission to the second layer was further reduced to 5.3% and 4.6% (GLM, $p < 0.001$) in comparison to 15% infected plants found in the second layer of the control.

I also conducted microcosm experiments with CMV-infected source plants and CMV-resistant Col-0 and CMV-resistant Ei-2 plants. I again found (Figure 5.15A) that more aphids settled on barriers of either CMV-resistant or susceptible Ei-2 plants within populations of Col-0 plants. The first layer of the control, only Col-0 plants, retained 31% of aphids, whereas 51% (GLM, $p < 0.001$) and 57% (GLM, $p < 0.001$) of aphids were trapped in barriers including susceptible or CMV-resistant Ei-2 plants (Figure 5.15A). CMV transmission in the second layer was reduced to 2% (GLM, $p < 0.01$) in barriers of CMV-resistant Ei-2 plants compared with 31% of plants infected in the second layer of the control microcosms (only Col-0 plants). Interestingly, CMV-infected plants in the second layer were equally reduced in microcosms containing mixtures of 33% of randomly distributed CMV-resistant or susceptible Ei-2 plants to 5% and 4%, respectively in comparison with 31% of infected plants found in the second layer in the control plants (GLM, $p < 0.001$).

Microcosm of Ei-2 plants and CMV-resistant plants showed similar results (Figure 5.16). However, as shown in Figure 5.16B in these microcosms only the addition of CMV-resistant Col-0 plants either as barriers or included as proportions of a randomised mixture with susceptible plants significantly reduced virus transmission in the first layer to 3% (GLM, $p < 0.05$) and to 7% (GLM, $p < 0.001$), respectively compared with 14% of CMV-infected plants found in the control microcosm (only Ei-2 plants). Interestingly, mixtures of 33% of CMV-resistant or susceptible Col-0 reduced virus transmission in the second layer to 3% (GLM, $p < 0.05$) and 2% (GLM, $p < 0.001$) compared with 15% of infected plants found in the control.

Taken together, these experiments suggest that barriers of aphid attractive, virus-resistant plants can trap and sanitise viruliferous aphids. Furthermore, randomised mixtures of virus-resistant with susceptible plants can reduce virus transmission as well.

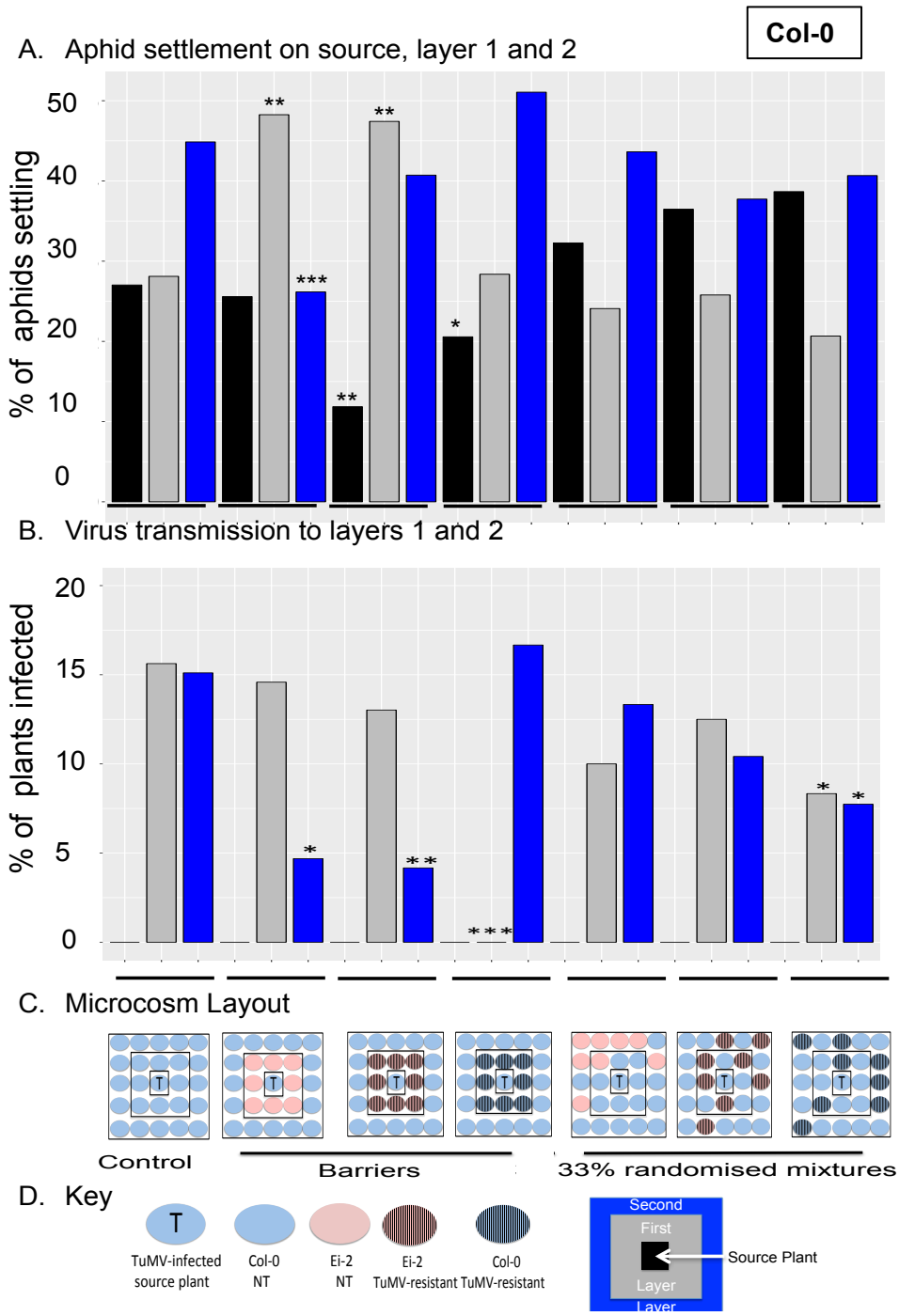


Figure 5.13 Barriers and 33% of randomised mixtures of plants in Col-0 populations reduce TuMV infection

TuMV-infected (10 dpi) source plants (infested with 30 aphids) were placed in the centre of 5x5 arrays (n = 8) (Figure 2.10). After 24 hours of aphid infestation, insecticide was applied. Arrays were kept for 2 weeks until TuMV symptoms appeared. In these experiments **Col-0** was the main crop and Ei-2 the trap plant. **A** shows the percentage of aphids found on the TuMV-infected source plant (black), first layer of plants (grey) and second layer of plants (blue). **B** shows the percentage of plants infected found on the first layer (grey) and second layer (blue). These experiments were performed in microcosm of **Col-0** plants. **C** and **D** Representations of microcosms layouts. Significant differences were determined between the control and each type of layout by a GLM *p < 0.05, **p < 0.01, ***p < 0.00. The percentages of aphids and infected plants found on each layer per treatment were compared with those found on each layer of the control.

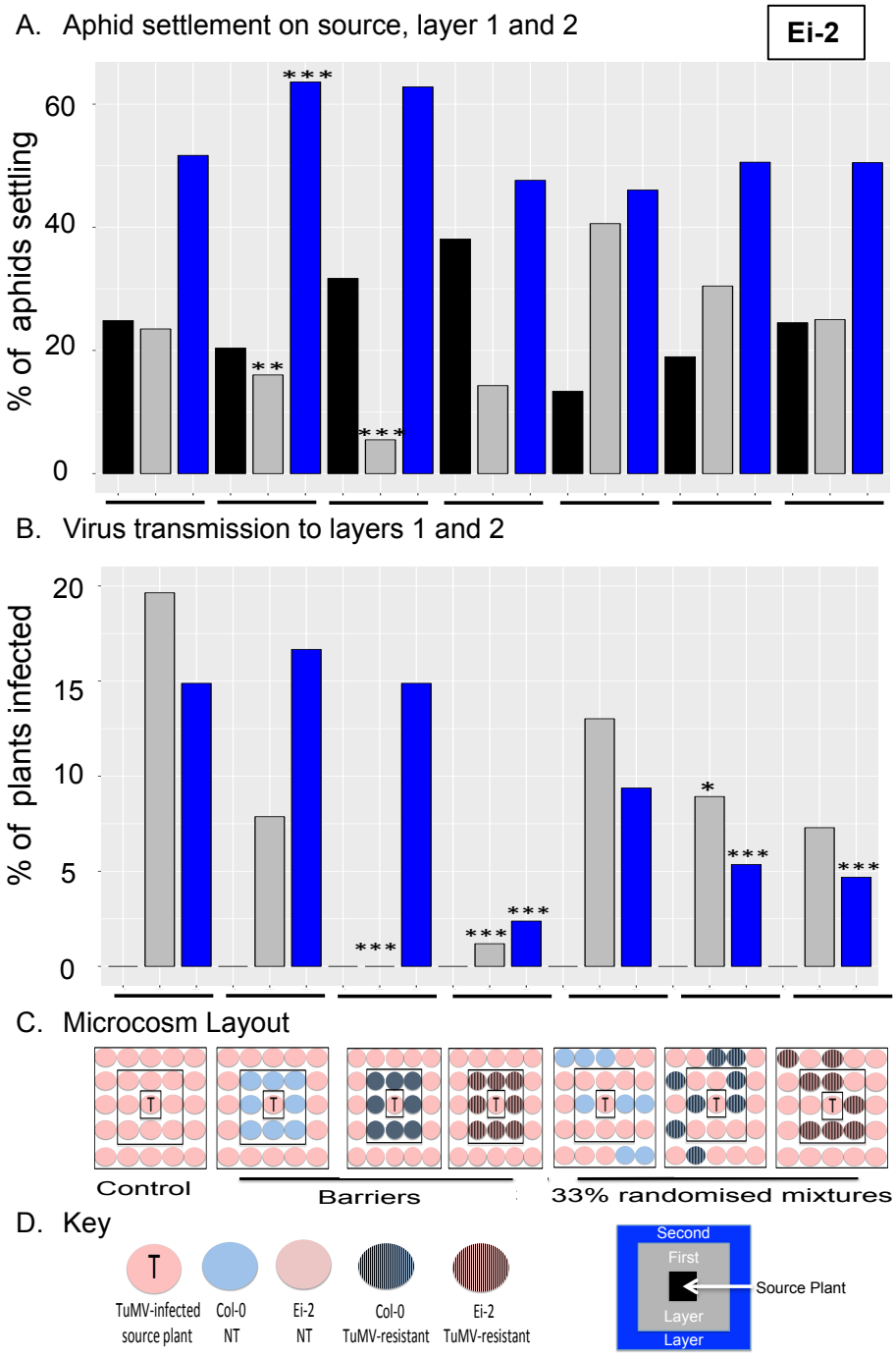


Figure 5.14 Barriers and 33% of randomised mixtures of plants in Ei-2 populations reduce TuMV infection

TuMV-infected (10 dpi) source plants (infested with 30 aphids) were placed in the centre of 5x5 arrays (n = 8) (Figure 2.10). After 24 hours of aphid infestation, insecticide was applied. Arrays were kept for 2 weeks until virus symptoms appeared. In these experiments microcosms of **Ei-2** plants were evaluated. **A** shows the percentage of aphids found on the TuMV-infected source plant (black), first layer of plants (grey) and second layer of plants (blue) **B** shows the percentage of TuMV-infected plants found on the first layer (grey) and second layer (blue) of each arrangement. **C** and **D** show microcosms layouts. Significant differences were determined between the control and each type of layout by a GLM *p < 0.05, **p < 0.01, ***p < 0.00. The percentages of aphids and infected plants found on each layer per treatment were compared with those found on each layer of the control.

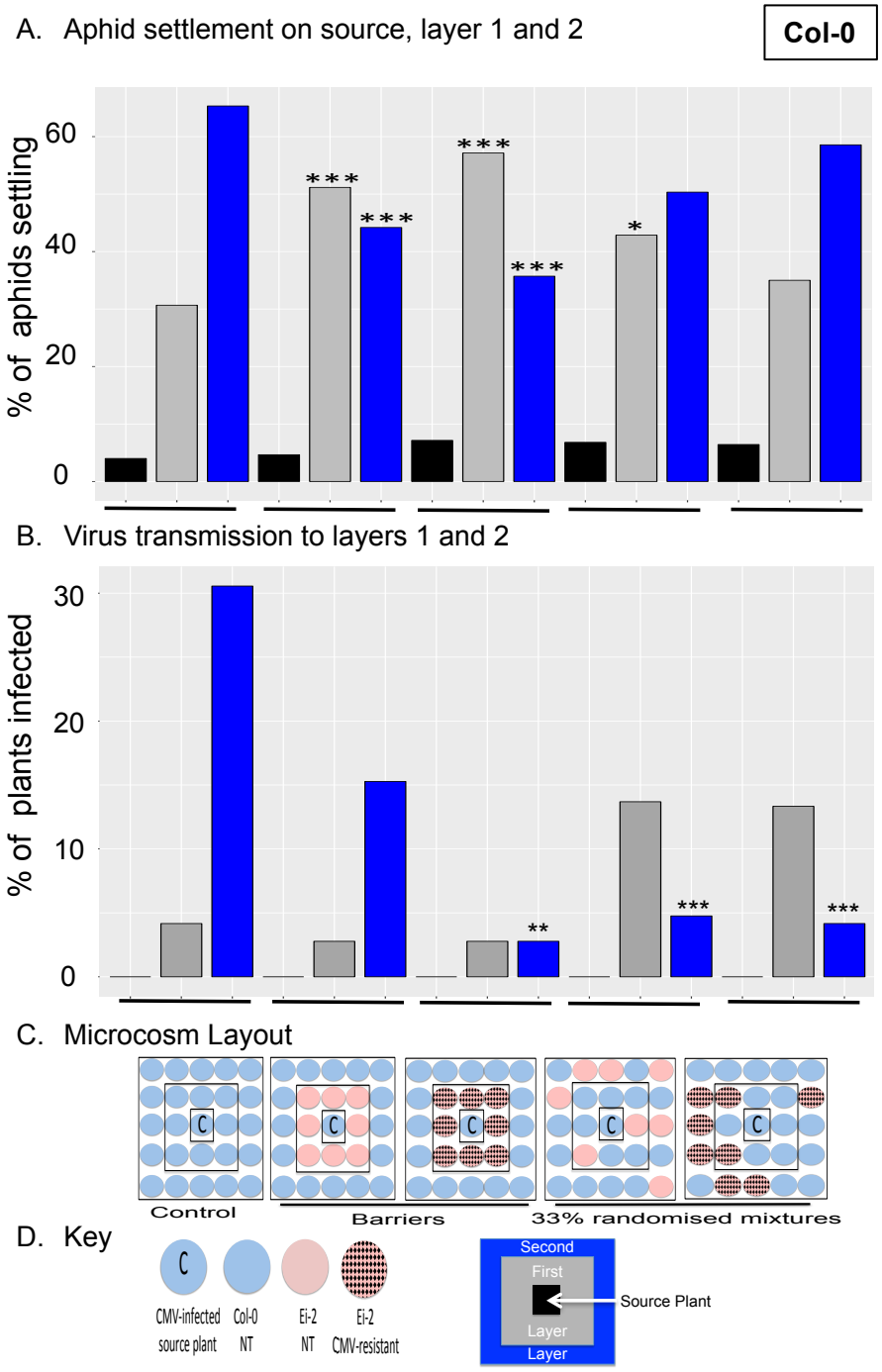


Figure 5.15 Barriers and 33% of randomised mixtures of plants in Col-0 populations reduce CMV infection

CMV-infected (10 dpi) source plants (infested with 30 aphids) were placed in the centre of 5x5 arrays (n = 8) (Figure 2.10). After 24 hours of aphid infestation, insecticide was applied. Arrays were kept for 2 weeks until virus symptoms appeared. These are microcosm experiments with **Col-0** plants. **A** shows the percentage of aphids found on the CMV-infected source plant (black), first layer of plants (grey) and second layer of plants (blue) **B** shows the percentage of CMV-infected plants found on the first layer (grey) and second layer (blue) of each arrangement. Significant differences were determined by a GLM *p < 0.05, **p < 0.01, ***p < 0.001. The percentages of aphids and infected plants found on each layer per treatment were compared with those found on each layer of the control.

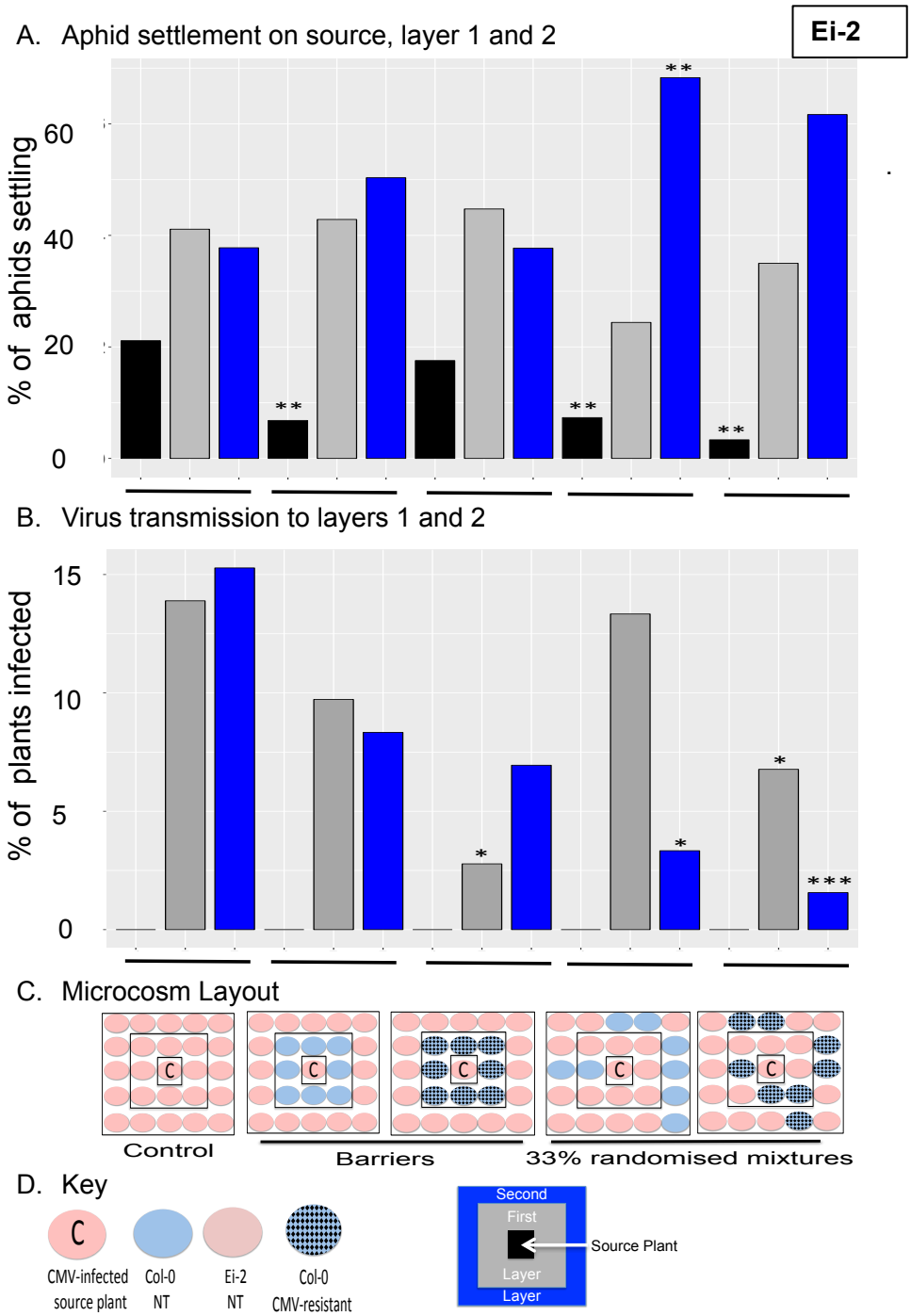


Figure 5.16 A barrier or 33% of CMV-resistant plants reduce CMV transmission by aphids in Ei-2 microcosms

CMV-infected (10 dpi) source plants (infested with 30 aphids) were placed in the centre of 5x5 arrays (n = 8). After 24 hours of aphid infestation, insecticide was applied. Arrays were kept for 2 weeks until CMV symptoms appeared. **A** shows the percentage of aphids found on the CMV-infected source plant (black), first layer of plants (grey) and second layer of plants (blue) **B** shows the percentage of CMV-infected plants found first layer (grey) and second layer (blue) of each arrangement. **C** and **D** show microcosms layouts Significant differences were determined by a GLM *p < 0.05, **p < 0.01, ***p < 0.001. The percentages of aphids and infected plants found on each layer per treatment were compared with those found on each layer of the control.

5.2.11 Mixing CMV-resistant and TuMV-resistant plants decreases the transmission of both viruses

I hypothesised that viruliferous aphids carrying both CMV and TuMV acquired from infected hosts with both viruses could be sanitised for one virus or the other or possibly for both. I arranged equal numbers of CMV-resistant plants and TuMV-resistant plants in either barriers or in randomised mixtures of 17% CMV-resistant and 17% TuMV-resistant plants. These microcosms had source plants infected with both viruses. Consistent with the results of Sections 5.2.7 and 5.2.10, I again found that aphids settled on Ei-2 barriers of plants placed in populations of Col-0 plants. As shown in Figure 5.17A, 63% of aphids (GLM, $p < 0.001$) were trapped on susceptible Ei-2 barriers and 57% of aphids (GLM, $p < 0.001$) were trapped on barriers containing CMV-resistant and TuMV-resistant Ei-2 plants compared with only 28% of aphids found on the first layer of plants in the control (only Col-0 plants). Furthermore, as depicted in Figure 5.17B virus transmission by aphids was significantly reduced when the Ei-2 barriers contained CMV-resistant and TuMV-resistant plants (GLM, $p < 0.05$). Interestingly, the proportion of plants that became infected with both viruses was reduced to 2% compared with 18% in the control (GLM, $p < 0.05$).

The use of barriers of CMV-resistant and TuMV-resistant plants did not reduce virus transmission of either virus or both in Ei-2 microcosms (Figure 5.18). Interestingly, as shown before with CMV or TuMV source plants in Ei-2 microcosms (Figure 5.14A and 5.16A), less aphids were found in a barrier of susceptible or virus-resistant Col-0 plants 7% and 23%, respectively compared with 35% aphids found in the first layer of the control. However, only randomised mixtures of CMV-resistant and TuMV-resistant Col-0 plants significantly reduced the rate of virus–infected plants to 6% (GLM, $p < 0.001$) (Figure 5.18 B). The results show that randomised mixtures

containing a 17% of CMV-resistant and 17% TuMV-resistant plants can reduce spread of mixed virus infections.

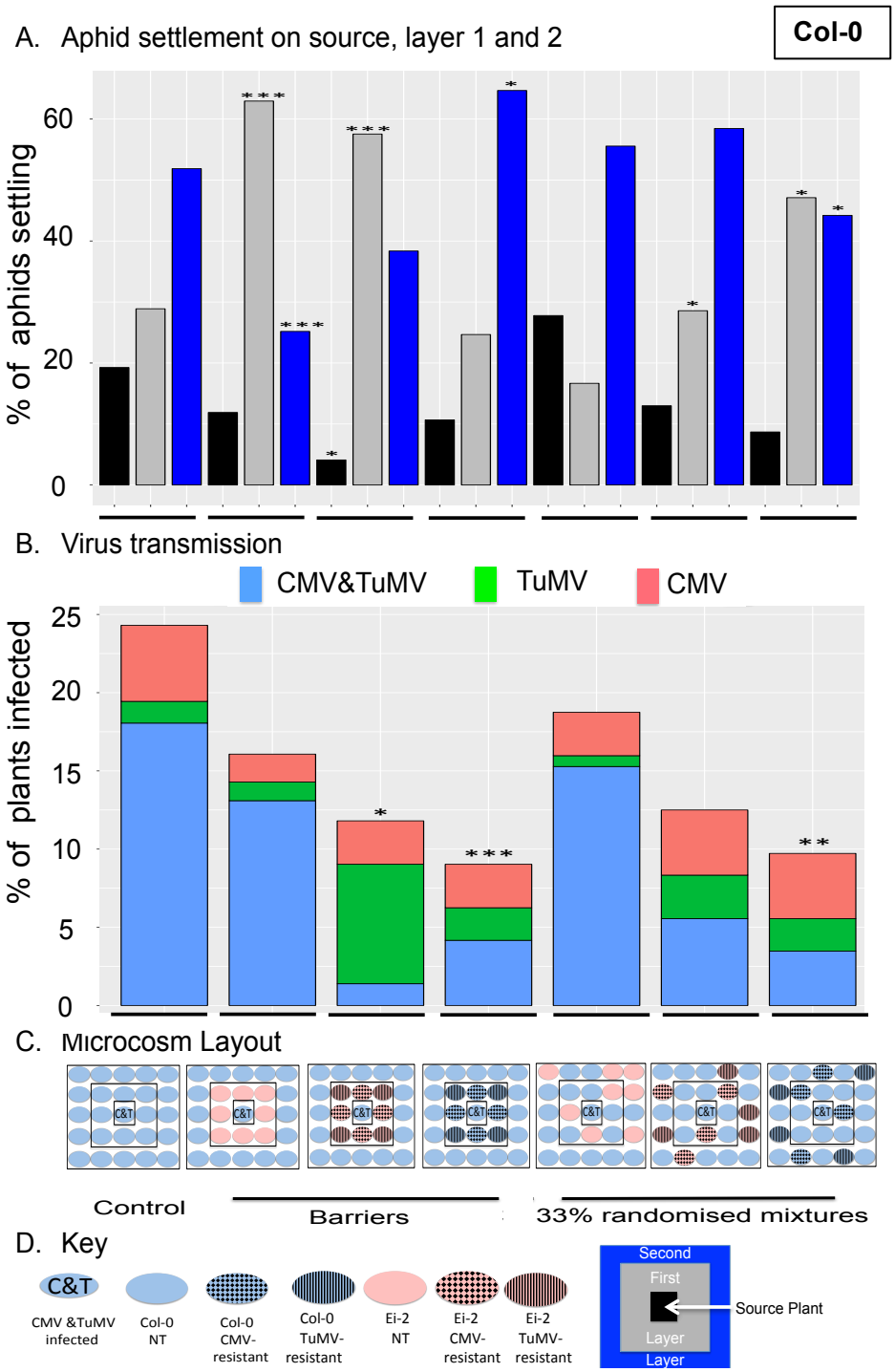


Figure 5.17 Mixtures of plants resistant to either CMV or TuMV reduced transmission of mixed virus inoculum by aphids

CMV- and TuMV-infected (10 dpi) source plants (infested with 30 aphids) were placed in the centres of 5x5 arrays (n = 8) (Figure 2.10). After 24 hours of aphid infestation, insecticide was applied. Arrays were kept for 2 weeks until virus symptoms appeared. In these experiments **Col-0** was the main crop and Ei-2 the trap plant. Half proportion of CMV and half proportion of TuMV-resistant plants were always used in arrangement containing virus-resistant plants **A** shows the

percentage of aphids found on the CMV- and TuMV-infected source plant (black), first layer of plants (grey) and second layer of plants (blue) **B** shows the percentage of virus-infected plants found on each arrangements. The proportion of symptomatic plants for CMV, TuMV and doubly infected with CMV and TuMV are depicted. **C** and **D** show microcosms layouts Significant differences were determined by a GLM *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. The percentages of aphids on each layer per treatment were compared with those found on each layer of the control. The percentage of infected plants found per array was compared with the control.

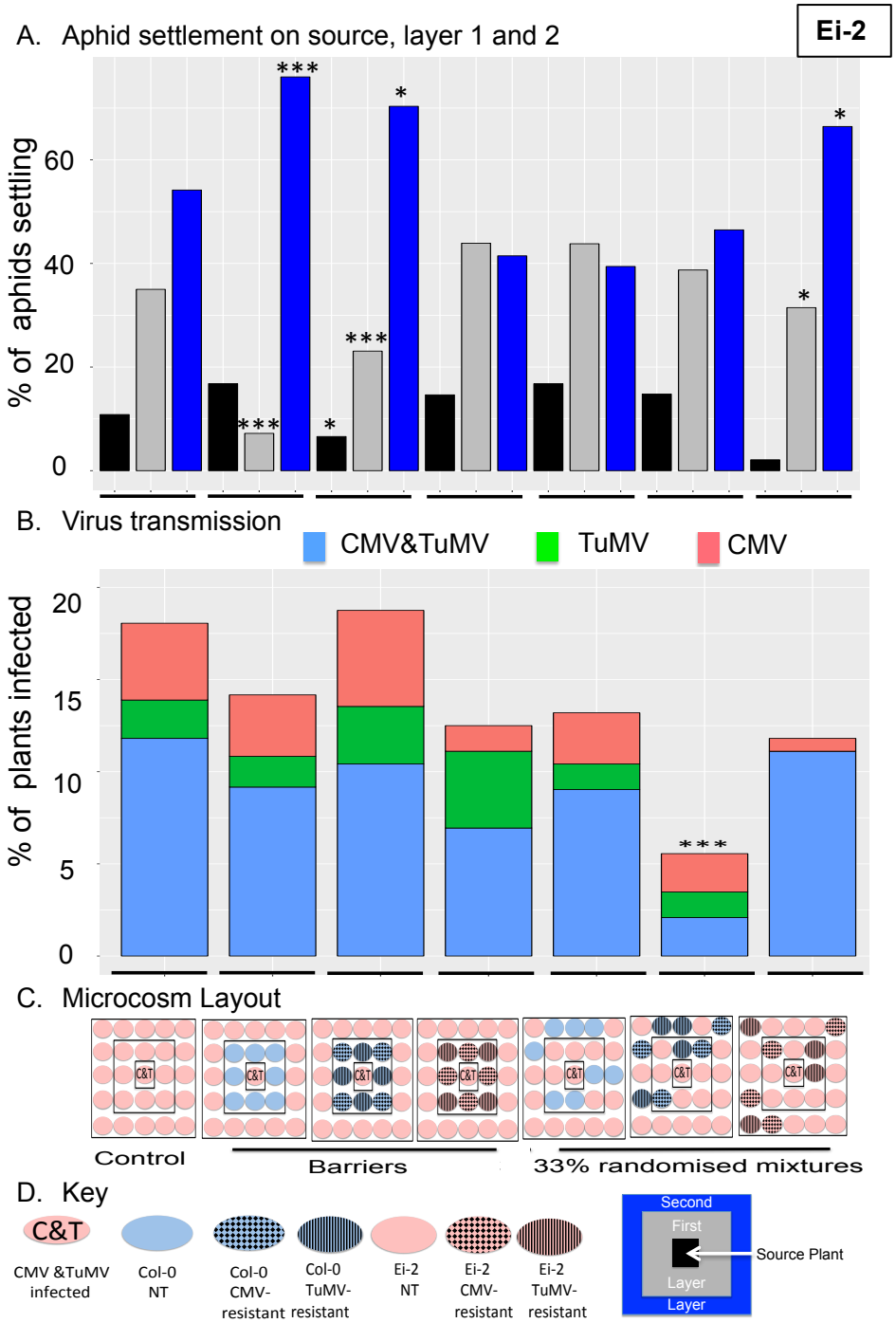


Figure 5.18 A mixture of 33% of TuMV- and CMV-resistant plants reduced mixed infections in Ei-2 populations

CMV and TuMV-infected (10 dpi) source plants (infested with 30 aphids) were placed in the centres of 5x5 arrays (n = 8). After 24 hours of aphid infestation, insecticide was applied. Arrays were kept for 2 weeks until virus symptoms appeared. In these experiments **Ei-2** was the main crop and Col-0 the trap plant. **A** shows the percentage of aphids found on the doubly CMV- and TuMV-infected source plant (black), first layer of plants (grey) and second layer of plants (blue) **B** shows the proportion of virus-infected plants found on arrangement. The proportion of CMV-,

TuMV- and doubly infected plants is shown. **C** and **D** show microcosms layouts Significant differences were determined by a GLM: *p < 0.05, **p < 0.01, ***p < 0.001. The percentages of aphids on each layer per treatment were compared with those found on each layer of the control. The percentage of infected plants found per array was compared with the control.

5.3 Discussion

5.3.1 CMV and TuMV induce repulsion of aphids as the viruses spread systemically through the plant

M. persicae had an equal preference for settling on CMV-infected or TuMV-infected Arabidopsis plants at early stages of infection. Thus, early in virus infection aphids can settle on virus-infected plants, which will allow aphid survival and reproduction. However, as the virus spreads systemically in the host, it becomes deterrent, which will promote dispersal of viruliferous aphids to new hosts. Thus, vector density may increase during early stages of infection that might result in more aphids available to transmit the virus once repulsion is induced. *M. persicae* was attracted at intermediate stages of infection to PLRV infected potato plants (Werner et al., 2009). However, PLRV is transmitted in persistent mode, which requires prolonged phloem feeding for virus acquisition and transmission. In my experiments, I studied non-persistently transmitted viruses, which have a short acquisition and inoculation period (Section 1.2). Thus, the time point of attraction to virus-infected plant may depend on virus strain and stage of virus infection as well as mode of virus transmission.

The results of my experiments looking at different parameters involved in virus transmission suggest that efficiency of virus transmission depends on the infectiousness of the source plant, vector density and movement of viruliferous aphids (Section 5.2.6). Together with the results of aphid settling choice tests (Section 5.2.1), the virus transmission experiments also suggest that the stage of virus infection influences virus spread to new hosts. These results further imply that a virus infected source plant reaches a very infectious stage, which is at 10dpi in Arabidopsis infected with CMV. Previous studies looking at the effect of virus infection on aphid behaviour and performance have shown that CMV induces the

emission of aphid attractive semiochemicals but also induces biosynthesis of feeding deterrent compounds (Mauck et al., 2010; Westwood et al., 2013a). In both studies experiments were performed after more than two weeks of virus infection. However, my results show a build-up of aphid deterrence, which suggest that virus-host-vector interactions might be more dynamic as virus-host-vector interactions might fluctuate throughout virus spread.

5.3.2 Aphids emigrate from CMV infected hosts

Aphids migrate away from CMV-infected plants of either Arabidopsis accession and the movement of viruliferous aphids caused virus spread to susceptible hosts. Interestingly, the combination of a highly deterrent host (Col-0) and a more aphid attractive plant (Ei-2) changes the dynamics of virus-vector-host interactions as aphid movement was altered (Figures 5.3). The fact that one accession (Ei-2) is more aphid attractive than the other (Col-0) indicates that one can exploit natural diversity to manipulate aphid behaviour and virus transmission. Aphid migration away from CMV-infected plants is in agreement with the proposed mechanisms to enhance transmission for viruses with non-persistent mode of transmission. This mechanism suggests that hosts infected with non-persistent viruses induce aphid deterrence which likely induce movement of aphids to new hosts and promote virus transmission (Mauck et al., 2010; Carmo-Sousa et al., 2014; Groen et al., 2017)

5.3.3 Viruliferous aphids can be lured to trap plants where they are sanitised

Virus transmission was successfully disrupted with the use of various proportions of virus-resistant plants in randomised mixtures or by using resistant plants as barriers. The results indicate that introducing small proportions of virus-resistant plants is an effective way to reduce virus spread. An even more effective strategy to disrupt virus transmission is the use of plants that are both aphid-attractive and virus-resistant. The results further suggest that microcosms can simulate field conditions to test plant virus disease management strategies.

Durable resistance can be broken when a pathogen mutates and overcome resistance (McDonald and Linde, 2002). Using small proportions of virus-resistant plants in randomised mixtures and as barriers may alleviate selection pressure for virus-resistance breaking virus mutants. Although viruses have more difficulty in overcoming genetic resistance (García-Arenal and McDonald, 2003), inhibiting resistance breakage is still advantageous and should be considered in order to alleviate selective pressures on the virus.

The strategies tested to manipulate aphid behaviour and virus transmission in Arabidopsis under controlled conditions might be a baseline for field experiments. The approaches could be extended to study virus secondary infection and how virus spread changes throughout the host lifecycle. The experiments were performed with synchronised age vectors and a single aphid species. In the future, one might want to try combinations of vectors to study how other conditions such as aphid species and aphid life stage might alter virus-host-vector dynamics. It would be also interesting to perform experiments with winged aphids as the experiments presented in this project were evaluated with wingless aphids, which are thought to be important for secondary spread (Raccah et al., 1985). It would be interesting to study how spatial movement of the virus by winged aphids changes the dynamics of virus spread.

Chapter 6 General discussion

6.1 Exploring the use of aphid attractive and virus-resistant decoy plants to inhibit aphid-mediated virus transmission

There is increasing evidence that insect transmitted viruses manipulate vector behaviour (Sections 1.9 and 6.4). The type of mode of transmission as well as vector efficiency might influence aphid-host-vector interactions (Section 1.9).

My focus was on viruses that are transmitted in the non-persistent manner by aphids. I explored combining knowledge of virus-host-vector interactions, especially of how viruses manipulate these relationships, to investigate methods to better control plant virus diseases using plant mixtures, e.g. mixtures including various proportions of aphid-attracting virus-resistant or susceptible plants in order to inhibit transmission. This may be a more sustainable means of controlling virus diseases in crops than simply planting a monoculture of resistant plants, since resistant monocultures may impose selection for resistance-breaking virus strains (Fraile and García-Arenal, 2018). Approaches using decoy plants are also compatible with the mixed cropping systems used in sub-Saharan Africa, and my project feeds into a wider effort to explore simple and sustainable methods to increase crop yields for resource-poor farmers. Despite this focus, however, I believe that 'decoying' approaches, such as the one I explored, could be applicable to any cropping system.

To establish my experimental approach I needed to find an amenable small-scale system for manipulating aphid movement between plants and make transgenic plants that could be proxies for resistant crop plants (transgenic or harbouring natural resistance genes). I have shown that Arabidopsis accessions display variation in their degree of attractiveness to the aphid *M. persicae* (Section 4.25) and that aphids settle more readily on the accession Ei-2 than to the accession Col-0 (Figure 4.3). I engineered virus resistance against CMV and TuMV in the Ei-2 accession to produce

plants that could both attract aphids and “trap” viral inoculum in non-permissive hosts (see Section 6.3). In Chapter 3, I described the generation of these CMV-resistant and TuMV-resistant plants in Col-0 and Ei-2 backgrounds. Candidate virus-resistant lines were assessed by mechanical and aphid-inoculation. Useful lines exhibited 70 to 100% resistance to either CMV or TuMV (Figure 3.8 and Table 3.3). These results show that inhibition of virus infection can vary between engineered virus-resistant lines. This is not a new observation as in the earliest studies of genetically engineered resistance to plant viruses it had been found that independent transgenic lines vary in terms of their resistance phenotype (Kaniewski and Thomas, 1999). However, not only completely resistant but also partly (70%) resistant lines could be successfully used to inhibit CMV and TuMV spread in microcosm experiments (Figures 5.11-5.15). I speculate that the aphids carrying the virus in on the acrostyle (Uzest, et al. 2007; Webster et al., 2018), which was acquired from virus-infected source plants in the microcosm experiments, carry variable quantities of viral inoculum on their stylets and that this may affect the extent of virus transmission. The CMV strain used in this project, Fny-CMV, shows a ‘cycling’ phenomenon which means that the virus is not distributed evenly throughout the plant and can vary in its concentration in plant tissue over time, so that leaves of an infected plant can have high or low virus titres at a given point (Gal-On et al., 1996).

6.2 CMV and TuMV induce host-vector interactions in an accession-specific manner

Viruses are obligate intracellular parasites that depend utterly on their host for replication and, in the case of most plant viruses, depend on vectors for transmission. How do plant viruses ensure their transmission? I found that CMV and TuMV induce emission of aphid-attractive volatiles by Arabidopsis and induce deterrence against aphid settling. This appears to be similar to the phenomenon first noted by Mauck and colleagues (2010) with squash plants infected with Fny-CMV.

My host location and olfactometry assays showed that *M. persicae* is also attracted to VOC blends emitted by CMV-infected and TuMV-infected Arabidopsis Col-0 and Ei-2 plants (Figures 4.9-4.11). However, migration experiments showed that when aphids were placed on CMV-infected Arabidopsis plants, aphids were encouraged to move away from CMV-infected plants (Figures 5.2 and 5.3). Aphid settling experiments at different time points of infection showed that both CMV and TuMV cause plants to become increasingly less favourable for aphid settlement as the viruses spread systemically in the host (Figure 6.1). The results suggest that both CMV and TuMV can induce combinations of biochemical changes in Arabidopsis that attract *M. persicae* but do not encourage aphid settling. It is not known if both viruses trigger exactly the same biochemical changes and in future work it would be interesting to examine this question. It is important to point out that at early stages of infection in my experiments, symptoms were not evident in virus-infected plants. Thus, aphid preference to settle on CMV- or TuMV infected plants at early times of infections might not be explained due to visual cues. Hodge and Powell (2008) reported that the pea aphid *A. pisum* preferred to settle on *V. faba* plants infected with pea enation mosaic virus (a circulative-persistent virus), bean yellow mosaic virus (a non-persistent virus) or bean mottle virus (a non-aphid transmissible virus). Interestengly, the pea aphid did not show an improved performance on the three types of virus-infected plants. However, in that study the authors used plants infected at 14-17 days post inoculation that were diagnosed by visual symptoms. Curiously, my work appears to contradict the observations of Casteel and colleagues (2014), who found that TuMV could induce susceptibility to aphid colonization in Arabidopsis (see next section).

6.3 Virus-induced effects of host-aphid interactions in different plant species

Plant viruses induce changes in plant emission of volatiles that affect host-vector interactions (Section 1.9). For example, the aphid *Rhopalosiphum padi* L. preferred to settle on wheat plants infected with barley yellow dwarf virus (BYDV). It was found that BYDV-infected plants emitted more volatiles than non-infected wheat plants (Jiménez-Martínez et al., 2004). Potato plants infected with PLRV arrested more aphids than non-infected potato plants. The preference of *M. persicae* for PLRV-infected potato plants was also attributed to the plant volatiles emitted by virus-infected plants (Eigenbrode et al., 2002). These studies suggested that plants infected with persistently transmitted viruses enhance attractiveness of these plants to vectors that may enhance retention of the vector for longer periods of time. Attraction and retention are critical for persistently transmitted viruses because the vectors of these viruses require sustained feeding for successful acquisition and transmission. After acquisition the insect vector remains viruliferous for life. Thus, attraction and retention might enhance virus transmission of persistently transmitted viruses.

However, in other virus-host-vector interactions, specifically viruses with non-persistent transmission, aphid performance and aphid behaviour may change depending on the host and virus strain. For example, on PVY-infected potato plants settling, growth and phloem feeding of *M. persicae* was enhanced compared to healthy potato plants (Srinivasan and Alvarez, 2007; Boquel et al., 2011). Studies of CMV-infected plants showed that CMV-infected squash, tomato and tobacco plants emit quantitatively higher VOCs than healthy plants (Mauck et al., 2010; Groen et al., 2016; Tungadi et al., 2017). However, increased emission of plant volatiles does not

always result in attraction of aphids to CMV-infected plants as seen with tobacco (Tungadi et al., 2017).

CMV-infected Arabidopsis Col-0 plants induce feeding deterrence affecting aphid performance in terms of growth and reproduction (Westwood et al., 2013a). Westwood and colleagues (2013a) showed that *M. persicae* encounters difficulties in sustaining phloem feeding in CMV-infected plants. My results are in agreement with those results as I also found that *M. persicae* experiences difficulties to reach the phloem on Col-CMV infected plants (Section 4.2.8). However, I found that CMV infection affects aphid performance in an accession specific manner in Arabidopsis. *M. persicae* growth and phloem feeding were similar in both CMV-infected and healthy Ei-2 plants (Figures 4.2 and 4.17). Interestingly, I found that aphids had difficulties reaching phloem in healthy Ei-2 plants. I speculate that the intrinsic differences between Col-0 and Ei-2 Arabidopsis accessions result in different host-virus vector dynamics. I showed that more aphids were attracted to Ei-2 plants but aphid growth rated and aphid feeding behaviour was had a negative effect. However, the negative effect seen in Ei-2 plants did not cause a decrease in aphid reproduction. Thus, suitable host plants may contain resistant features but this resistance does not affect the overall susceptibility. These findings seem to indicate that virus-infection is host-specific. In other hosts, such as tobacco, CMV infection enhances aphid reproduction and survival but not aphid growth (Ziebell et al., 2011).

TuMV infection also induces different responses in host-vector interactions depending on the host species (Casteel et al., 2014, 2015; Bak et al., 2017). Casteel et al (2014) found that TuMV infection promotes aphid reproduction on both Arabidopsis Col-0 and *N. benthamiana* plants. However, aphids preferred to settle on TuMV-infected *N. benthamiana* than on healthy plants. Although Casteel at el (2014) did not show choice test experiments with TuMV-infected Arabidopsis, their results

with *N. benthamiana* differ with my results with TuMV-infected Arabidopsis Col-0 and Ei-2 plants. I found that aphids do not prefer to settle on TuMV-infected Arabidopsis Col-0 and Ei-2 plants at later stages of infection. The results suggest that TuMV also induces changes in aphid performance and behaviour depending on the host species. However, Casteel and colleagues (2014) did not specify the time post-infection on their aphid performance and aphid behaviour experiments. As shown in my experiments the time post-infection have different responses on aphid behaviour. Thus, in order to draw accurate conclusions, it would be ideal to compare results with experiments performed under similar conditions. In addition, virus-host-vector interactions might also be different depending on the vector studied. Chesnais et al (2017) reported that TuYV infection or CaMV infection induces positive or negative effects on aphid performance and behaviour, and virus-mediated effect depends on the virus transmission mode as well as transmission efficiency. Taken together, the results suggest that CMV and TuMV infection induce changes in host-vector interactions in a host-specific manner. However, the variability can be found within a species as well. The different responses observed in varieties of the same species as shown in this study, raises the possibility of using this knowledge to manipulate aphid behaviour to inhibit virus spread by mixing varieties with opposite host-vector interactions (see Section 6.4). I also speculate that viruses manipulate host olfactory and/or gustatory cues to promote virus spread but it might depend on host genotype, time point of virus spread in the host, aphid density, and aphid age (Section 5.2.6).

6.4 Future prospects: Mixing aphid-attractive plants and virus-resistant plants inhibited virus transmission by aphids

Spread of non-persistently transmitted viruses can be rapid because aphids do not need to settle and feed on plants for a prolonged time to acquire or inoculate virus particles. The results showed that mixtures of Col-0 and Ei-2 plants alter aphid

behaviour. Also, the addition of virus-resistant plants into microcosm experiments reduces virus transmission by aphids. The spatial distribution of virus spread of CMV and TuMV suggests that wingless aphids used in my experiments transmitted both viruses to adjacent plants. The results are in agreement with the spatial distribution reported for CMV-infected melons *(Cucumis melo)*, which was studied under field conditions (Alonso-Prados et al., 2003). It was found that CMV spread by aphids followed a rectangular pattern. It was suggested that the pattern of spread depends on the ability of the vector to colonize melons (Alonso-Prados et al., 2003). It would be interesting to further study the spatial distribution of CMV and TuMV spread when winged aphids are introduced in the microcosms as well as non-colonizing aphids, although this may be technically challenging in my microcosm design.

Intercropping is a farming method that has been commonly used in small-scale farms (Hooks and Fereres, 2006). The main benefits of intercropping includes low cost inputs due to limited mechanization needed and pest control (Brooker et al., 2015). Border or trap crops to inhibit virus transmission have previously been proposed as a potential management tool of plant virus transmission (Hooks and Fereres, 2006). Possible drawbacks of using trap or crop barriers include secondary virus disease spread and the use of intraspecific plants used competing with the main crop (Section 1.10). In previous studies, the border or trap crop assessed to inhibit virus transmission of non-persistently transmitted virus was a crop of different species to the main crop. For example, barriers of sorghum (*Sorghum vulgare)* and maize (*Zea mays)* were used to protect pepper (*Capsicum annuum)* plants against PVY and CMV (Fereres, 2000). Fereres (2000) attributed the reduction of virus transmission to the height of barrier of plants, which acted as decoys to sanitise viruliferous aphids. It was suggested that ideal plant barrier should be non-host for the virus and the vector, but attractive for aphid landing.

In my experiments I tackled these potential challenges by using mixtures of virus-resistant plants and plant accessions of the same species. Resistance to pathogens can be improved by using mixtures of susceptible and resistant varieties in field experiments. For example, rice blast infection was reduced by 94% when mixtures of susceptible and resistant plants were planted compared with genetic monocultures (Zhu et al., 2000). The use of 50% powdery mildew-resistant wheat plants had 32% lower powdery-mildew infection than monocultures of non-transgenic plants (Zeller 2012). In my experiments, I used a constant aphid density thereby a reduction of aphid population could not be assessed. However, I assessed aphid movement and found that the mixtures of plant accessions changed aphid behaviour (Figures 5.11-5.15). Thus, these studies and my experiments show that proportions of pathogen-resistant plants can reduce the spread of pathogen infection. In future experiments, it would be interesting to see whether the free movement of winged aphids into the mixed plant microcosm experiments alters aphid density and virus spread.

Trap plants may harbour high aphid densities, which could potentially initiate secondary virus spread (Hooks and Fereres, 2006). High aphid populations induce the emission of plant volatiles that attract aphid parasitoids (Girling et al., 2006). The aphid parasitoid *Diaeretiella rapae* is attracted to volatiles emitted by Arabidopsis plants infested with *M. persicae* (Girling et al., 2006). Girling and colleagues (2006) suggested that parasitoids have stronger responses to heavier aphid infestations. Thus, in the context of my microcosm experiments, trap plants that support high aphid infestation would increase aphid parasitoid responses to locate aphid-infested hosts. Since the trap plants are also virus-resistant it is less likely that aphid movement would promote secondary virus spread. To rule out this potential issue, in future experiments I would recommend assessing secondary spread of viruses.

My results showed the addition of virus-resistant plants to microcosms reduces virus transmission by aphids. How could these findings be applied? First, in places where the introduction of genetically modified plants is not allowed (or in cases where crops and plants are not easily transformable), identification and deployment of aphid attractive crop lines could be an interim approach to disrupt insect-mediated virus transmission until breeding programs develop virus-resistant plants. Trap crops could have both olfactory signals to attract vectors and resistance to viruses to inhibit virus spread in the crop. As shown in my experiments (Section 5.2.10), the additive effect is a more efficient method to reduce virus transmission by aphids.

The use of barriers and small proportions of traps plants indicate two other possible uses. These are: (i) When the virus source is known, one can recommend isolating the source of infection with a barrier of virus-resistant plants to inhibit the movement of viruliferous aphids to new hosts. (ii) From an agricultural point of view, using a proportion of virus-resistant plants is more advantageous than barrier planting, as plant mixtures do not depend on a specific arrangement. Furthermore, the rapid development of genetically modified lines and current techniques such as CRISPR-Cas9 can be used to develop plant lines with additive “traits” which will enhance plant pathogen and pest management. For example, gene editing has been used to engineer plants with recessive resistance to potyviruses (Chandrasekaran et al., 2016; Pyott et al., 2016). As more information on plant genes controlling aphid-plant interactions emerges, it may be possible to ‘edit’ plants to increase the attraction of vectors to virus-resistant crop plant.

6.5 Future prospects: Understanding virus-host-vector interactions to develop approaches for virus control

I showed that virus infection induces specific host/variety-vector interactions (Section 6.3). CMV and TuMV induce aphid deterrence in Arabidopsis plants, and this

deterrence is magnified as virus spreads systemically in the host (Figure 5.1). How then does the virus maintain aphid populations if virus infection in the host is causing aphid deterrence? I propose that the efficiency of aphid-mediated transmission of non-persistent viruses depends on virus spread in the host, which controls aphid behaviour and aphid performance (Figure 6.1). I found that, at early time-points of virus infection, aphids are not deterred and aphid reproduction is not affected. However, as virus disease progresses, plant deterrence against aphids increases and aphids are encouraged to leave the infected host and transmit the virus to new hosts. Thus, the virus not only increases its spread but also encourages the increase of its aphid vector population and its host persistence. It has been shown that virus infection improves host tolerance to abiotic stress such as drought (Xu et al., 2008; Westwood et al., 2013b), and cold (Xu et al., 2008; Fernández-Calvino et al., 2014).

Viruses may also pay back to their hosts by attracting pollinators, which are also influenced by multiple plant factors including nutritional and energetic rewards and volatile and visual cues that might be affected by virus infection (Bailes et al., 2018; Groen et al., 2016; Netsai Mhlanga Pers. Comm). Groen and colleagues (2016) found that CMV-infected tomato plants emit plant volatiles that alter foraging behaviour of the bumblebee, *Bombus terrestris*. CMV-infected tomato plants have a reduced seed yield. However, an increase in seed yield was reported when flowers of CMV-infected plants were bee-pollinated. Overall, understanding the progress of virus diseases and how they affect the host and the vector offer new avenues to explore the management of viral diseases. I speculate that under field conditions, for example in a crop, the interactions are more complex, whereby virus source hosts at different time points of infection are simultaneously present. Therefore, the management of virus inhibition in the field should be carried out along with early detection of virus (Sankaran et al., 2010) as at early stages of virus infection vector populations might be high yet symptoms not evident.

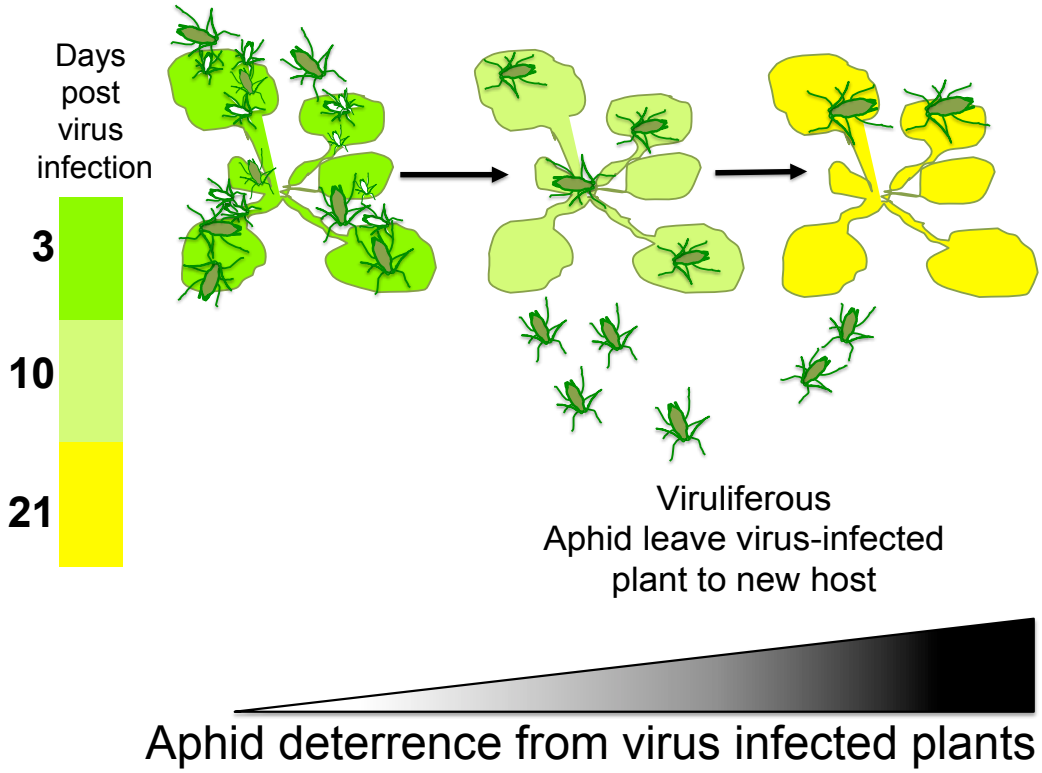


Figure 6.1 Illustration of virus-host-vector interactions over the course of virus spread in the host

At early time points virus infection in the host does not induce aphid deterrence. Thus, insect vectors (aphids) can reproduce. However, as the virus diseases spread systemically in the host, aphid deterrence increases. The number of aphids (adults and nymphs) decreases as virus infection progresses. Virus infection induces aphid deterrence to encourage viruliferous aphids to move and spread the virus to new hosts. Virus-infected plants are arranged from the left to the right according to disease progression. Adult aphids are represented in light green and nymphs are represented in white. The colour of each plant represents the days post infection according to the legend as follows: green, 3 days post-infection (3dpi), light green 10 days post-infection (10dpi), and yellow 21 days post-infection (21dpi). The degree of aphid deterrence is represented by the width of the wedge.

6.6 Future perspectives on inhibition of plant virus transmission

Taken together, the research on virus-plant-aphid interactions from model plants such as Arabidopsis can be translated into approaches to control plant diseases (Section 6.4). Intercropping of plants of the same species or different species is considered a sustainable approach particularly for smallholder farmers (Boudreau, 2013). Although intercropping requires integration of planting dates, and fertilization, it has been shown that intercropping improves yields and control pests on small-

scale farms in sub-Saharan Africa (Brooker et al., 2015; Pickett and Khan, 2016). A stimulating example is the 'Push-Pull' field design, which is an integrative approach for pest management that has been successful in reducing stem borer pest damage in cereals such as maize and sorghum in sub-Saharan cereal systems (Pickett and Khan, 2016).

The development and successful application of the push-pull technology relied on the exploitation of insect host selection behaviour. Leptidopterous stem borers are repelled from crops by, for example, *Desmodium spp.* plants ("push") and are concentrated on attractive trap plants, for example, Napier grass ("pull)" plant (*Melinis minutiflora*) (Cook et al., 2007; Pickett and Khan, 2016). The push-pull system has been improved over recent years from a pest control system in different cereal crops to a platform that can achieve weed control, plant nutrition and forage for animal husbandry (Pickett and Khan, 2016). Thus, wider exploitation of a management tool can be achieved. This, in the context of insect-mediated transmission of plant viruses requires more understanding of the molecular mechanisms behind virus-host-vector interactions. In my project, I showed that aphid behaviour could be exploited to manipulate virus transmission. Although experiments are under controlled conditions and use model plants rather than crop plants, microcosm experiments with Arabidopsis have the advantage of being quicker than experimenting on annual crops in the field. In addition, one can evaluate several approaches and define the most relevant and efficient to then be evaluated under field conditions.

Variation in olfactory signals between aphids species and their host has been reported (Bruce et al., 2005). The ability of plant viruses to alter and manipulate vector behaviour has now been reported in several studies using different viruses, hosts and vectors (Section 6.3) (Mauck, 2016; Groen at al., 2018). Both olfactory

signals and virus manipulation were used in my findings. I made use of this knowledge and improved on previous work using trap/barrier crops to manipulate aphid location cues. I reported that the use of aphid attractive lines as well as virus-resistant plants is an original strategy to disrupt transmission of non-persistent viruses. Although it is known that virus-resistant plants are effective in inhibiting virus spread not only in model plants but also in crops (Lindbo and Falk, 2017), the use of randomised small proportions of virus-resistant plants that are also aphid attractive is novel. The integration of virus resistance and olfactory stimuli when viruliferous aphids locate a host plant was successful to disrupt the transmission of non-persistently transmitted viruses.

However, for other types of viruses, for example those with persistent transmission, the use of attractive lines would increase virus spread since viruliferous aphids carry the virus for their lifespan, unless those lines also possessed resistance to the virus. In these scenarios, one would suggest using virus-resistant and insect attractive lines that are “dead-end” hosts (i.e. attractive to the insect but the insect cannot survive). Thus, there are opportunities to further investigate how my findings could be used to manage viruses with different modes of transmission. Future work is needed to determine whether my strategies are effective under field conditions where aphid populations and sources of virus infection are variable. In conclusion, the manipulation of aphid behaviour based on studies of virus-host-vector interactions offers tools to disrupt virus transmission. Future work is needed to identify semiochemicals and host genes involved in changes of aphid behaviour and performance induced by virus infection as they will be new means to manipulate aphid behaviour and virus transmission. Finding novel approaches to manage viral diseases is critical for controlling diseases that threaten food security especially in regions where resource-poor farmers are in need of more cost effective approaches.

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Appendices

Appendix 1: List of primers

Forward primer denotes that the sequence primes extension of the sense DNA strand. Reverse primer denotes that the sequence primes extension of the antisense DNA strand.

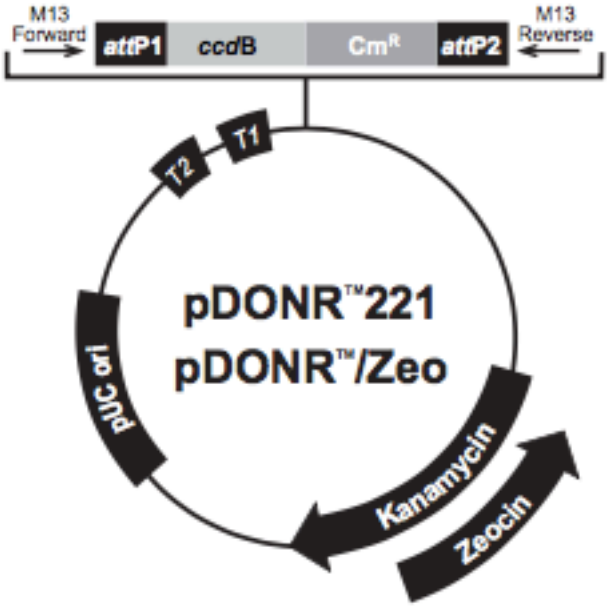
Primer	Name	Direction	Sequence 5'-3'	Use
1	2b amplification	Forward	AAAAAGCAGGCTTACTGGCTCGTATGGTGGAGG	Amplify 2b sequence region and add half *att* sites
2	2b amplification	Reverse	AGAAAGCTGGGTTGCCACGTTCACATGGCGG	Amplify 2b sequence region and add half *att* sites
3	Adapter for full *att* sites	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCT	Gateway cloning
4	Adapter for full *att* sites	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGT	Gateway cloning
5	Amplify PIPO sequence region and add half *att* sites	Forward	AAAAAGCAGGCTTAATGGCAGAACCAAACTGG	Gateway cloning
6	Amplify PIPO sequence region and add half *att* sites	Reverse	AGAAAGCTGGGTTCTCCGTTCGTAAGATGACATG	Gateway cloning
7	HP-2b	Reverse	AAAAAGCAGGCTTACTGGCTCGTATGGTGGAGG	Used with primer 8
8	Intron binary vector pKGWIWG2(II)0	Forward	CATATACCAGTTAACGTGTCTC	To locate intron in pKGWIWG2(II)0 plasmid
9	HP-PIPO	Reverse	CTTAATGGCAGAACCAAACT	Used with primer 8
10	M13	Forward	GTAAAACGACGGCCAG	pDONR221
11	M13	Reverse	CAGGAAACAGCTATGAC	pDONR221
12	hptII (Hygromycin B)	Forward	AGTCAATGACCGCTGTTATGCG	Selection marker
13	hptII (Hygromycin B)	Reverse	ACAGCGTCTCCGACCTGATGCA	Selection marker
14	RB	Reverse	CTTAGGTTTACCCGCCAATATA	Right border of T-DNA
15	35S	Forward	GGAAGGTCTTGCGAAGGATAG	35S promoter
16	Kan	Forward	TGACTCTAGCTAGAGTCCGAACCC	Selection marker
17	Kan	Reverse	CACCAAGCGAAACATCGCAT	Selection marker
18	amiRNA	Forward	GGCTAAAATGGAAAGTCGTGG	To detect insertion of ami-SD-3
19	amiRNA	Reverse	AGTCAGCACCCCCGATTTTACC	To detect insertion of ami-SD-3

Appendix 2: Plasmid maps
Gateway donor/vector pDONR 221 (INVITROGEN)

Map and Features of pDONR™221 and pDONR™/Zeo

Map of pDONR™221 and pDONR™/Zeo

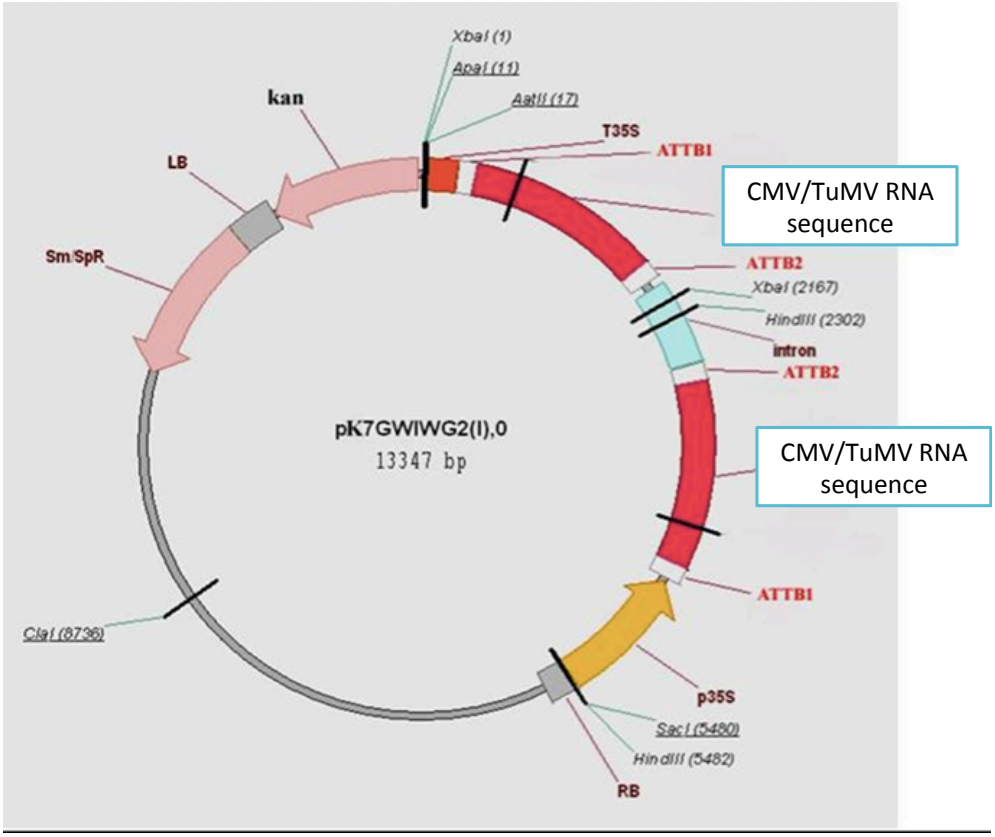
The following map shows the elements of pDONR™221 and pDONR™/Zeo. The complete sequences of pDONR™221 and pDONR™/Zeo are available from www.lifetechnologies.com or by contacting Technical Support (page 17).



Comments for:	pDONR™221 4761 nucleotides	pDONR™/Zeo 4291 nucleotides
*rrn*B T2 transcription termination sequence (c):	268-295	268-295
rrnB T1 transcription termination sequence (c):	427-470	427-470
M13 Forward (-20) priming site:	537-552	537-552
*att*P1:	570-801	570-801
*ccd*B gene (c):	1197-1502	1197-1502
Chloramphenicol resistance gene (c):	1825-2505	1847-2506
*att*P2 (c):	2753-2984	2754-2985
M13 Reverse priming site:	3026-3042	3027-3043
Kanamycin resistance gene:	3155-3964	---
EM7 promoter (c):	---	3486-3552
Zeocin resistance gene (c):	---	3111-3485
pUC origin:	4085-4758	3615-4288

(c) = complementary strand

Gateway destination vector pK7GWIWG2(II)0



Appendix 3: Summary table for aphid and mechanical assessment of virus-resistant lines

Plant	Line	Construct	Inoculum	Mechanical inoculation		Aphid inoculation		
				Number of plants inoculated	Proportion of plants resistant to virus	Number of 3x3 arrays	Total number of plants	Proportion of plants resistant to virus
Arabidopsis Col-0	5.17	ami-SD-3	CMV	20	80%	5	40	75%
	1.12	ami-SD-3	CMV	20	80%	5	40	70%
	6.8	ami-SD-3	CMV	20	15%	5	40	40%
	10.34	HP-2b	CMV	20	100%	4	32	88%
	3.12	HP-2b	CMV	19	84%	4	32	84%
	2.42	HP-2b	CMV	18	94%	4	32	91%
	1.19	HP-PIPO	TuMV	20	100%	4	32	100%
	5.53	HP-PIPO	TuMV	17	100%	4	32	100%
	3.1	HP-PIPO	TuMV	20	100%	4	32	100%
Arabidopsis Ei-2	3.38	ami-SD-3	CMV	20	60%	3	24	81%
	6.26	ami-SD-3	CMV	20	50%	3	24	75%
	7.1	ami-SD-3	CMV	20	80%	3	24	88%
	1.13	HP-2b	CMV	15	87%	3	24	83%
	10.3	HP-2b	CMV	20	85%	3	24	75%
	11.13	HP-2b	CMV	20	88%	3	24	83%
	1.23	HP-PIPO	TuMV	15	73%	3	24	75%
	2.15	HP-PIPO	TuMV	17	71%	3	24	71%
N. benthamiana	27	HP-PIPO	TuMV	15	100%	3	24	92%
	W2(4)	HP-PIPO	TuMV	15	100%	3	24	100%
	Y2(13)	HP-PIPO	TuMV	15	100%	3	24	100%

Appendix 4 Summary table of ami-SD-3 lines assessed by mechanical inoculatation

Plant	Line	Construct	Inoculum	Mechanical inoculation	
				Number of plants inoculated	Proportion of plants resistant to virus
Arabidopsis Col-0	2.5	ami-SD-3	CMV	20	40%
	4.7	ami-SD-3	CMV	19	26%
	8.2	ami-SD-3	CMV	18	11%
	3.4	ami-SD-3	CMV	20	10%
	3.1	ami-SD-3	CMV	17	35%
	8.2	ami-SD-3	CMV	19	21%
Arabidopsis Ei-2	1.22	ami-SD-3	CMV	20	15%
	2.19	ami-SD-3	CMV	20	20%
	4.5	ami-SD-3	CMV	20	10%